


JC07 Rec'd PCT/PTO 07 JAN 2002

FORM PTO-1390 (Modified) (REV 5-93)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				065691-0267	
				U.S. APPLICATION NO. (If known, see 37 CFR 1.51) Unassigned <b>10/019954</b>	
INTERNATIONAL APPLICATION NO. PCT FR00/01972		INTERNATIONAL FILING DATE 07/07/2000		PRIORITY DATE CLAIMED 07/07/1999	
TITLE OF INVENTION METHOD FOR PRODUCING OLIGOPOLYSACCHARIDES					
APPLICANT(S) FOR DO/EO/US SAMAIN, Eric, PRIEM, Bernard					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1.	<input checked="" type="checkbox"/>	This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.			
2.	<input type="checkbox"/>	This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.			
3.	<input type="checkbox"/>	This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).			
4.	<input type="checkbox"/>	A proper Demand for International Preliminary Examination was made by the 19 <sup>th</sup> month from the earliest claimed priority date.			
5.	<input checked="" type="checkbox"/>	A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). <input checked="" type="checkbox"/> has been transmitted by the International Bureau. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)			
6.	<input type="checkbox"/>	A translation of the International Application into English (35 U.S.C. 371(c)(2)).			
7.	<input checked="" type="checkbox"/>	Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). <input type="checkbox"/> have been transmitted by the International Bureau. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. <input checked="" type="checkbox"/> have not been made and will not be made.			
8.	<input type="checkbox"/>	A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).			
9.	<input type="checkbox"/>	An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).			
10.	<input type="checkbox"/>	A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).			
11.	<input type="checkbox"/>	Applicant claims small entity status under 37 CFR 1.27 .			
Items 12. to 17. below concern other document(s) or information included:					
12.	<input type="checkbox"/>	An Information Disclosure Statement under 37 CFR 1.97 and 1.98.			
13.	<input type="checkbox"/>	An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.			
14.	<input type="checkbox"/>	A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.			
15.	<input type="checkbox"/>	A substitute specification.			
16.	<input type="checkbox"/>	A change of power of attorney and/or address letter.			
17.	<input checked="" type="checkbox"/>	Other items or information: Application Data Sheet			

JC13 Rec'd PCT/PTO 07 JAN 2002

U.S. APPLICATION NO. (If known, see 37 CFR 1.55) Unassigned <b>10/019954</b>		INTERNATIONAL APPLICATION NO. PCT/FR00/01972		ATTORNEY'S DOCKET NUMBER 065691-0267	
18. <input checked="" type="checkbox"/> The following fees are submitted:				<b>CALCULATIONS</b>	
Basic National Fee (37 CFR 1.492(a)(1)-(5): Search Report has been prepared by the EPO or JPO.....\$890.00					
International preliminary examination fee paid to USPTO (37 CFR 1.482).....\$710.00					
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) .....\$740.00					
Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$1,040.00					
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) .....\$100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$890.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than 20 Months from the earliest claimed priority date (37 CFR 1.492(e))				\$0.00	
Claims	Number Filed	Included in Basic Fee	Extra Claims	Rate	
Total Claims	46	20	26	\$18.00	
Independent Claims	3	3	0	\$84.00	
Multiple dependent claim(s) (if applicable)				\$280.00	
TOTAL OF ABOVE CALCULATIONS =				\$1358.00	
Reduction by 1/2 for filing by small entity, if applicable.				\$0.00	
SUBTOTAL =				\$1358.00	
Processing fee of \$130.00 for furnishing English translation later the 20 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$130.00	
TOTAL NATIONAL FEE =				\$1488.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +					
TOTAL FEES ENCLOSED =				\$1488.00	
				Amount to be: refunded \$	
				charged \$	
a. <input checked="" type="checkbox"/> A check in the amount of \$1488.00 to cover the above fees is enclosed.					
b. <input type="checkbox"/> Please charge my Deposit Account No. <u>19-0741</u> in the amount of \$0.00 to the above fees. A duplicate copy of this sheet is enclosed.					
c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>19-0741</u> . A duplicate copy of this sheet is enclosed.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:			<div style="display: flex; justify-content: space-between;"> <div> <b>Foley &amp; Lardner</b>  Customer Number: 22428    <b>22428</b>  PATENT TRADEMARK OFFICE </div> <div> <div style="text-align: right;"> <i>Michael D. Kaminski</i>  Michael D. Kaminski  SIGNATURE <i>Reg. No. 32,404, for</i> </div> <hr/> NAME STEPHEN B. MAEBIUS  <hr/> REGISTRATION NUMBER 35,264 </div> </div>		

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

10/019954

Applicant: Eric SAMAIN et al  
Title: METHOD FOR PRODUCING  
OLIGOPOLYSACCARIDES  
Appl. No.: 10/019,954  
Filing Date: 05/24/2002  
Examiner: Unassigned  
Art Unit: Unassigned

**PRELIMINARY AMENDMENT**

Commissioner for Patents  
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application, Applicants respectfully request that the following amendments be entered into the application:

**In the Claims:**

Please substitute for original claims 3, 5, 6, 8, 10, 13, 14, 17, 25, 27, 29, 30, 34-42, 45 and 46 the following rewritten versions of the same claims, as amended. The changes are shown explicitly in the attached "Versions With Markings to Show Changes Made."

3. (Amended) The method as claimed in claim 1, characterized in that said cell is a cell chosen from bacteria and yeasts.

5. (Amended) The method as claimed in claim 1, characterized in that said modification is chosen from glycosylation, sulfatation, acetylation, phosphorylation, succinylation, methylation, and addition of an enolpyruvate group.

6. (Amended) The method as claimed in claim 1, characterized in that said enzyme is an enzyme capable of performing a glycosylation, chosen from glycosyl-transferases.

8. (Amended) The method as claimed in claim 1, characterized in that said cell culturing is carried out on a carbon-based substrate.

10. (Amended) The method as claimed in claim 8, characterized in that said culturing is performed under conditions allowing the production of a culture with a high cell density.

13. (Amended) The method as claimed in claim 11, characterized in that said precursor is added during phase b).

14. (Amended) The method as claimed in claim 1, characterized in that said precursor is of carbohydrate nature, preferably of oligosaccharide nature.

17. (Amended) The method as claimed in claim 15, for the production of ( $\beta$ -D-Gal-[1  $\rightarrow$  4]- $\beta$ -D-GlcNac-1 $\rightarrow$ O-allyl), characterized in that

- said cell is a bacterium of *LacZ*<sup>-</sup> genotype;
- said enzyme is  $\beta$ -1, 4-galactosyl-transferase;
- said substrate is glycerol;
- said precursor is allyl-N-acetyl- $\beta$ -D-glucosaminide ( $\beta$ -D-GlcNac-1 $\rightarrow$ O-allyl).

20. (Amended) The method as claimed in claim 18, characterized in that said active transport of said precursor is performed by lactose permease.

25. (Amended) The method as claimed in claim 1, characterized in that said cell lacks any enzymatic activity liable to degrade said precursor(s).

27. (Amended) The method as claimed in claim 1, characterized in that it also comprises the addition of an inducer to said culture medium to induce the expression in said cell of said enzyme and/or of a protein involved in said active transport.

29. (Amended) The method as claimed in claim 1, for the production of the trisaccharide 4-O-[3-O- (2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl) - $\beta$ -D-galactopyranosyl] -D-glucopyranose, ( $\beta$ -D-GlcNac-[1 $\rightarrow$ 3] - $\beta$ -D-Gal-[1 $\rightarrow$ 4] -D-Glc), characterized in that:

- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype;
- said enzyme is  $\beta$ -1, 3-N-acetyl-glucosaminyl-transferase;
- said substrate is glycerol;
- said inducer is isopropyl  $\beta$ -D-thiogalactoside (IPTG);
- said precursor is lactose.

30. (Amended) The method as claimed in claim 1, for the production of lacto-N-neo-tetraose and polylactosamine (lacto-N-neo-hexaose, lacto-N-neo-octaose, lacto-N-neo-decaose), characterized in that:

- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype;
- said enzymes are  $\beta$ -1, 3-N-acetyl-glucosaminyl-transferase and  $\beta$ -1, 4-galactosyl-transferase;
- said substrate is glucose;
- said inducer is isopropyl- $\beta$ -D-thiogalactoside (IPTG);
- said precursor is lactose.

34. (Amended) The method as claimed in claim 1, for the production of 3'-sialyllactose ( $\alpha$ -NeuAc-[2 $\rightarrow$ 3] -  $\beta$ -D-Gal-[1 $\rightarrow$ 4] -  $\beta$ -D-Glc) or 6'-sialyllactose ( $\alpha$ -NeuAc-[2 $\rightarrow$ 6] -  $\beta$ -D-Gal-[1 $\rightarrow$ 4] - $\beta$ -D-Glc), characterized in that:

- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup>, *NanA* or *NanT*<sup>+</sup> genotype;
- said enzymes are CMP-NeuAc-synthase and  $\alpha$ -2, 3-sialyl-transferase or  $\alpha$ -2, 6-sialyl-transferase;
- said substrate is glycerol;
- said inducer is isopropyl- $\beta$ -D-thiogalactoside (IPTG);
- said precursors are lactose and sialic acid.

35. (Amended) The method as claimed in claim 1, for the production of 3'-fucosyllactose ( $\beta$ -D-Gal-[1 $\rightarrow$ 4]-( $\alpha$ -L-Fuc-[1 $\rightarrow$ 3]-D-Glc) or 2'-fucosyllactose,  $\alpha$ -L-Fuc-

[1→2] -β-D-Gal- [1→4] -D-Glc characterized in that it comprises a said enzyme chosen from α-1, 3-fucosyl-transferase or α-1, 2-fucosyl-transferase, and in that the cell has a *wcaJ<sup>-</sup> lacZ<sup>-</sup>* genotype and overexpresses the *rcsA* gene and in that said precursor is lactose.

36. (Amended) The method as claimed in claim 1, for the production of allyl 3-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl) -β-D-galactopyranoside, (β-D-GlcNac-[1→3] -β-D-Gal-1→O-allyl), characterized in that:

- said cell is a bacterium of *LacZ<sup>-</sup>, LacY<sup>+</sup>* genotype;
- said enzyme is β-1, 3-N-acetyl-glucosaminyl-transferase;
- said substrate is glycerol;
- said inducer is isopropyl β-D-thiogalactoside (IPTG);
- said precursor is allyl-β-D-galactopyranoside.

37. (Amended) The method as claimed in claim 1, for the production of analogs of lacto-N-neo-tetraose and of polylactosamines in which the glucose residue is replaced with an allyl group, characterized in that

- said cell is a bacterium of *LacZ<sup>-</sup>, LacY<sup>+</sup>* genotype;
- said enzymes are β-1,3-N-acetyl-glucosaminyl-transferase and β-1,4-galactosyl-transferase;
- said substrate is glucose;
- said inducer is isopropyl β-D-thiogalactoside (IPTG);
- said precursor is allyl-β-D-galactopyranoside.

38. (Amended) The method as claimed in claim 31, for the production of oligosaccharide analogs in which the glucose residue is replaced with an allyl group, characterized in that said precursor is allyl-β-D-galactoside.

39. (Amended) The method as claimed in claim 1, for producing an oligosaccharide labeled with at least one isotope, characterized in that said cell is cultured on said carbon-based substrate labeled with said isotope and/or in the presence of a said precursor labeled with said isotope.

40. (Amended) An oligosaccharide which may be obtained by the method as claimed in claim 1.

41. (Amended) An oligosaccharide which may be obtained by the method as claimed in claim 17, characterized in that the double bond of the allyl group of said oligosaccharides is chemically modified by addition, oxidation or ozonolysis reactions to form activated oligosaccharides that may be used for the chemical synthesis of glycoconjugates or glycopolymers.

42. (Amended) The oligosaccharide as claimed in claim 40, as a medicinal product.

45. (Amended) A pharmaceutical composition, characterized in that it comprises an oligosaccharide as claimed in claim 42 and a pharmaceutically acceptable vehicle.

46. (Amended) The agricultural or agronomic use of an oligosaccharide as claimed in claim 40, especially for the growth and defense of plants.

**REMARKS**

Applicant respectfully request that the foregoing amendments to Claims 3, 5, 6, 8, 10, 13, 14, 17, 25, 27, 29, 30, 34-42, 45 and 46 be entered in order to avoid this application incurring a surcharge for the presence of one or more multiple dependent claims.

Respectfully submitted,

Date May 24, 2002

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**Versions With Markings to Show Changes Made**

3. (Amended) The method as claimed in claim 1 [or 2], characterized in that said cell is a cell chosen from bacteria and yeasts.

5. (Amended) The method as claimed in [one of claims 1 to 4] claim 1, characterized in that said modification is chosen from glycosylation, sulfatation, acetylation, phosphorylation, succinylation, methylation, and addition of an enolpyruvate group.

6. (Amended) The method as claimed in [one of claims 1 to 5] claim 1, characterized in that said enzyme is an enzyme capable of performing a glycosylation, chosen from glycosyl-transferases.

8. (Amended) The method as claimed in [any one of claims 1 to 7] claim 1, characterized in that said cell culturing is carried out on a carbon-based substrate.

10. (Amended) The method as claimed in [one of claims 8 and 9] claim 8, characterized in that said culturing is performed under conditions allowing the production of a culture with a high cell density.

13. (Amended) The method as claimed in [either of claims 11 and 12] claim 11, characterized in that said precursor is added during phase b).

14. (Amended) The method as claimed in [one of claims 1 to 13] claim 1, characterized in that said precursor is of carbohydrate nature, preferably of oligosaccharide nature.

17. (Amended) The method as claimed in [either of claims 15 and 16] claim 15, for the production of ( $\beta$ -D-Gal-[1  $\rightarrow$  4]- $\beta$ -D-GlcNac-1 $\rightarrow$ O-allyl), characterized in that

- said cell is a bacterium of *LacZ* genotype;
- said enzyme is  $\beta$ -1, 4-galactosyl-transferase;

- said substrate is glycerol;
- said precursor is allyl-N-acetyl- $\beta$ -D-glucosaminide ( $\beta$ -D-GlcNac-1 $\rightarrow$ O-allyl).

20. (Amended) The method as claimed in [either of claims 18 and 19] claim 18, characterized in that said active transport of said precursor is performed by lactose permease.

25. (Amended) The method as claimed in [any one of claims 1 to 24] claim 1, characterized in that said cell lacks any enzymatic activity liable to degrade said precursor(s).

27. (Amended) The method as claimed in [any one of claims 1 to 26] claim 1, characterized in that it also comprises the addition of an inducer to said culture medium to induce the expression in said cell of said enzyme and/or of a protein involved in said active transport.

29. (Amended) The method as claimed in [any one of claims 1 to 28] claim 1, for the production of the trisaccharide 4-O-[3-O- (2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl) - $\beta$ -D-galactopyranosyl] -D-glucopyranose, ( $\beta$ -D-GlcNac-[1 $\rightarrow$ 3] - $\beta$ -D-Gal-[1 $\rightarrow$ 4] -D-Glc), characterized in that:

- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype;
- said enzyme is  $\beta$ -1, 3-N-acetyl-glucosaminyl-transferase;
- said substrate is glycerol;
- said inducer is isopropyl  $\beta$ -D-thiogalactoside (IPTG);
- said precursor is lactose.

30. (Amended) The method as claimed in [any one of claims 1 to 28] claim 1, for the production of lacto-N-neo-tetraose and poly lactosamine (lacto-N-neo-hexaose, lacto-N-neo-octaose, lacto-N-neo-decaose), characterized in that:

- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype;
- said enzymes are  $\beta$ -1, 3-N-acetyl-glucosaminyl-transferase and  $\beta$ -1, 4-galactosyl-transferase;

- said substrate is glucose;
- said inducer is isopropyl- $\beta$ -D-thiogalactoside (IPTG);
- said precursor is lactose.

34. (Amended) The method as claimed in [any one of claims 1 to 28] claim 1, for the production of 3'-sialyllactose ( $\alpha$ -NeuAc-[2 $\rightarrow$ 3] -  $\beta$ -D-Gal-[1 $\rightarrow$ 4] -  $\beta$ -D-Glc) or 6'-sialyllactose ( $\alpha$ -NeuAc- [2 $\rightarrow$ 6] -  $\beta$ -D-Gal-[1 $\rightarrow$ 4] - $\beta$ -D-Glc), characterized in that:

- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup>, *NanA* or *NanT*<sup>+</sup> genotype;
- said enzymes are CMP-NeuAc-synthase and  $\alpha$ -2, 3-sialyl-transferase or  $\alpha$ -2, 6-sialyl-transferase;
- said substrate is glycerol;
- said inducer is isopropyl- $\beta$ -D-thiogalactoside (IPTG);
- said precursors are lactose and sialic acid.

35. (Amended) The method as claimed in [any one of claims 1 to 28] claim 1, for the production of 3'-fucosyllactose ( $\beta$ -D-Gal-[1 $\rightarrow$ 4]-( $\alpha$ -L-Fuc-[1 $\rightarrow$ 3]-D-Glc) or 2'-fucosyllactose,  $\alpha$ -L-Fuc- [1 $\rightarrow$ 2] - $\beta$ -D-Gal- [1 $\rightarrow$ 4] -D-Glc characterized in that it comprises a said enzyme chosen from  $\alpha$ -1, 3-fucosyl-transferase or  $\alpha$ -1, 2-fucosyl-transferase, and in that the cell has a *wcaJ lacZ*<sup>-</sup> genotype and overexpresses the *rcaA* gene and in that said precursor is lactose.

36. (Amended) The method as claimed in [any one of claims 1 to 28] claim 1, for the production of allyl 3-O-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl) - $\beta$ -D-galactopyranoside, ( $\beta$ -D-GlcNac- [1 $\rightarrow$ 3] - $\beta$ -D-Gal-1 $\rightarrow$ O-allyl), characterized in that:

- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype;
- said enzyme is  $\beta$ -1, 3-N-acetyl-glucosaminyl-transferase;
- said substrate is glycerol;
- said inducer is isopropyl  $\beta$ -D-thiogalactoside (IPTG);
- said precursor is allyl- $\beta$ -D-galactopyranoside.

37. (Amended) The method as claimed in [any one of claims 1 to 28] claim 1, for the production of analogs of lacto-N-neo-tetraose and of polylactosamines in which the glucose residue is replaced with an allyl group, characterized in that

- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype;
- said enzymes are  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase and  $\beta$ -1,4-galactosyl-transferase;
- said substrate is glucose;
- said inducer is isopropyl  $\beta$ -D-thiogalactoside (IPTG);
- said precursor is allyl- $\beta$ -D-galactopyranoside.

38. (Amended) The method as claimed in [one of claims 31 to 35] claim 31, for the production of oligosaccharide analogs in which the glucose residue is replaced with an allyl group, characterized in that said precursor is allyl- $\beta$ -D-galactoside.

39. (Amended) The method as claimed in claim[s] 1 [to 38], for producing an oligosaccharide labeled with at least one isotope, characterized in that said cell is cultured on said carbon-based substrate labeled with said isotope and/or in the presence of a said precursor labeled with said isotope.

40. (Amended) An oligosaccharide which may be obtained by the method as claimed in [any one of claims 1 to 39] claim 1.

41. (Amended) An oligosaccharide which may be obtained by the method as claimed in [any one of claims 17, 19, 36 and 37] claim 17, characterized in that the double bond of the allyl group of said oligosaccharides is chemically modified by addition, oxidation or ozonolysis reactions to form activated oligosaccharides that may be used for the chemical synthesis of glycoconjugates or glycopolymers.

42. (Amended) The oligosaccharide as claimed in claim 40 [or 41], as a medicinal product.

45. (Amended) A pharmaceutical composition, characterized in that it comprises an oligosaccharide as claimed in [any one of claims 42 to 44] claim 42 and a pharmaceutically acceptable vehicle.

46. (Amended) The agricultural or agronomic use of an oligosaccharide as claimed in claim 40 [or 41], especially for the growth and defense of plants.

METHOD FOR PRODUCING OLIGOSACCHARIDES

The present invention relates to the or even  
5 microbiological production of oligosaccharides of  
biological interest.

It is now well-established that oligosaccharides play  
an important biological role especially as regards the  
10 activity and function of proteins; thus, they serve to  
modulate the half-life of proteins, and occasionally  
they are involved in the structure of the protein.  
Oligosaccharides play an essential role in antigen  
variability (for example blood groups), and in certain  
15 bacterial infections such as those caused by *Neisseria*  
*meningitidis*.

As oligosaccharides are usually obtained in a low yield  
by purification starting from natural sources, the  
20 synthesis of oligosaccharides has become a major  
challenge of carbohydrate chemistry, so as to supply  
sufficient amounts of well-characterized oligosac-  
charides, required for fundamental research or for any  
other potential applications (Boons et al., 1996).

25 The synthesis of complex oligosaccharides of biological  
interest may be performed chemically, enzymatically or  
microbiologically.

30 Despite the development of new chemical methods for  
synthesizing oligosaccharides in the course of the last  
20 years, the chemical synthesis of oligosaccharides  
remains very difficult on account of the numerous  
selective protection and deprotection steps, the  
35 lability of the glycoside linkages, the difficulties in  
obtaining regiospecific couplings, and the low  
production yields. As the number of steps increases  
with the size of the oligosaccharide, the preparation

of large quantities of oligosaccharides longer than trisaccharides is no simple matter. Contrary to the experience of peptide synthesis or nucleic acid synthesis, traditional synthetic organic chemistry cannot at the present time provide a high-quality and large-quantity synthesis of oligosaccharides, even of simple formula.

Consequently, the enzymatic methods have become more popular since they allow a regioselective synthesis under mild conditions and without a step for protection of the hydroxyl groups. The development of the enzymatic approach was made possible by the cloning and functional identification of numerous genes encoding the enzymes involved in the synthetic pathway of oligosaccharides. Thus, various types of enzyme may be used for the *in vitro* synthesis of oligosaccharides. The physiological function of the glycosyl-hydrosylases and of the glycosyl-phosphorylases is to depolymerize the oligosaccharides, but they may also be used *in vitro* in the synthesis of oligosaccharides by controlling the reaction equilibrium and kinetics. The substrates of the enzymes for these reactions are readily available, but these enzymatic reactions are not very versatile. Another enzymatic method developed uses the glycosyl-transferases of the Leloir biochemical pathway, which show strong regiospecificity for the precursor and also for the donor substrate; these glycosyl-transferases are not as readily available as the glycosyl-hydrolases. The recombinant DNA technique has recently made it possible to clone and produce a certain number of them. However, the main limitation of this enzymatic method lies in the very high cost of the sugar-nucleotides that are the sugar donors used by these enzymes.

The microbiological route for producing recombinant oligosaccharides *in vivo* is the most appealing of the synthetic routes since the bacterium is simultaneously

responsible for the biosynthesis of the enzymes, the regeneration of the sugar-nucleotides and, finally, the production of the oligosaccharide.

- 5 The first descriptions of the microbiological synthesis of oligosaccharides using recombinant bacteria may be considered to a certain extent as the studies which led to the elucidation of the pathways for the biosynthesis of the nodulation factors; these factors are signal  
10 molecules secreted by the rhizobia to allow recognition by leguminous plants in the nodulation process. Nodulation factors consist of a chito-oligosaccharide backbone bearing various substitutions. The functional identification of the *nod* genes involved in the  
15 biosynthesis of the nodulation factors was partly performed by identifying the oligosaccharides formed *in vivo* in strains of *Escherichia coli* expressing these various *nod* genes (G  r  mia et al., 1994; Kamst et al., 1995; Spaink et al., 1994; Mergaert et al., 1995).  
20 However, the production of oligosaccharides per se was not the aim of these studies; these products were synthesized only in trace amounts and were identified only by means of using radioactive precursors.
- 25 On the other hand, it was recently demonstrated in our laboratory (Samain et al., 1997) that the culturing at high cell density of *Escherichia coli* strains containing the *nodC* (chito-oligosaccharide synthase) gene made it possible to produce large amounts, of  
30 greater than 2 g/l, of "recombinant" chito-oligosaccharides.

However, this technique of microbiological synthesis of oligosaccharides remains limited to the production only  
35 of chito-oligosaccharides, due to the unique property of *nodC* (chito-oligosaccharide synthase) of functioning without a precursor; specifically, the other enzymes glycolyze a specific precursor and their activity is thus dependent on the presence of this precursor in the



cell. The problem of the precursor is thus the main obstacle blocking the development of the method and its extension to the production of other types of oligosaccharide.

5

One subject of the present invention is thus a method for producing an oligosaccharide of interest by a genetically modified cell starting with at least one exogenous precursor internalized by said cell, said precursor being involved in the biosynthetic pathway of said oligosaccharide, said method comprising the steps (i) of obtaining a cell that comprises at least one recombinant gene encoding an enzyme capable of modifying said exogenous precursor or one of the intermediates in the biosynthetic pathway of said oligosaccharide from said exogenous precursor necessary for the synthesis of said oligosaccharide from said precursor, and also the components for expressing said gene in said cell, said cell lacking any enzymatic activity liable to degrade said oligosaccharide, said precursor and said intermediates; (ii) of culturing said cell in the presence of at least one said exogenous precursor, under conditions enabling the internalization according to a mechanism of passive and/or active transport of said exogenous precursor by said cell and the production of said oligosaccharide by said cell.

According to one particular embodiment, the present invention relates to a method as described above, characterized in that said cell also comprises at least one gene encoding an enzyme capable of modifying an endogenous precursor involved in the biosynthetic pathway of said oligosaccharide, said enzyme being identical to or different than the enzyme used in the method described above, and also to the components for expressing said gene in said cell and characterized in that said cell lacks any enzymatic activity liable to degrade said precursor.

The term "oligosaccharides" is intended to denote linear or branched polymers with a variable number of residues, linkages and subunits; the number of residues being greater than 1. Oligosaccharides are carbohydrates that become converted on hydrolysis into several monosaccharide molecules; the monosaccharides being sugars that cannot be converted into a simpler substance by hydrolysis. Monosaccharides are subdivided into trioses, tetroses, pentoses, hexoses and heptoses depending on the number of carbon atoms in their hydrocarbon-based chain, and also into aldoses and ketoses depending on the presence of an aldehyde function or a ketone function in their molecule. Among the monosaccharides that are most frequently encountered, mention may be made of mannose, glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine. The number of chains of stereoisomeric oligosaccharides is extremely large, due to the large number of asymmetric carbons in the hydrocarbon-based chain.

The expression "exogenous precursor" is intended to denote a compound involved in the biosynthetic pathway of the oligosaccharide according to the invention that is internalized by said cell. The expression "endogenous precursor" is intended to denote a compound involved in the biosynthetic pathway of the oligosaccharide according to the invention that is naturally present in said cell.

The expression "genetically modified cell" is intended to denote a microorganism in which at least one alteration of the DNA sequence has been introduced into its genome in order to give said cell a particular phenotype. Such alterations may thus, for example, give the cell the ability not to degrade or not to modify a compound according to the invention, or not to reduce the DNA rearrangement frequency.

The method according to the invention is characterized in that said cell is a cell chosen from bacteria and yeasts. According to one preferred embodiment of the invention, the bacterium is chosen from the group composed of *Escherichia coli*, *Bacillus subtilis*, *Campylobacter pylori*, *Helicobacter pylori*, *Agrobacterium tumefaciens*, *Staphylococcus aureus*, *Thermophilus aquaticus*, *Azorhizobium caulinodans*, *Rhizobium leguminosarum*, *Neisseria gonorrhoeae* and *Neisseria meningitis*. According to one preferred embodiment of the invention, the bacterium is *Escherichia coli*. According to another embodiment of the invention, the cell is a yeast that is preferably *Saccharomyces cerevsae*, *Saccharomyces pombe* or *Candida albicans*. The cell according to the invention lacks any enzymatic activity liable to degrade said oligosaccharide, said precursor or said metabolic intermediates.

The nucleic acid sequence encoding the enzyme according to the invention is either naturally present in said cell or is introduced into said cell by the recombinant DNA techniques known to those skilled in the art. In the present description, the term "nucleic acid" will be intended to denote a DNA fragment, which is either double-stranded or single-stranded, or products of transcription of said DNAs, and/or an RNA fragment. According to one preferred embodiment, the nucleic acid sequence which is introduced into said cell by the recombinant DNA techniques and which encode an enzyme involved in the biosynthetic pathway of the oligosaccharide of interest is heterologous. The expression "heterologous nucleic acid sequence" is intended to denote a nucleic acid sequence that is not naturally present in the cell according to the invention. The heterologous nucleic acid sequence according to the invention may originate from any

animal or plant, eukaryotic or prokaryotic cell type and may originate from viruses.

Among the prokaryotic cells from which the heterologous  
5 nucleic acid sequence originates, mention should be made of bacteria and in particular *Escherichia coli*, *Bacillus subtilis*, *Campylobacter pylori*, *Helicobacter pylori*, *Agrobacterium tumefaciens*, *Staphylococcus aureus*, *Thermophilus aquaticus*, *Azorhizobium*  
10 *caulinodans*, *Rhizobium leguminosarum*, *Rhizobium meliloti*, *Neisseria gonorrhoeae* and *Neisseria meningitis*.

Among the unicellular eukaryotic cells from which the  
15 heterologous nucleic acid sequence originates, mention should be made of yeasts and in particular *Saccharomyces cerevisiae*, *Saccharomyces pombe* and *Candida albicans*.

20 According to one preferred embodiment, the heterologous nucleic acid sequence originates from plant or animal eukaryotic cells. According to an even more preferred embodiment, the heterologous nucleic acid sequence originates from mammalian cells and preferably from  
25 human cells.

According to one preferred embodiment of the invention, the cell according to the invention is the bacterium *Escherichia coli* and the nucleic acid sequence  
30 introduced into the bacterium and encoding the enzyme according to the invention preferably originates from a bacterium chosen from the group mentioned above.

According to one preferred embodiment of the invention,  
35 the nucleic acid sequence encoding the enzyme according to the invention is introduced into said cell in the form of an expression vector. The vector must comprise a promoter, translation start and stop signals, and also regions suitable for regulating transcription. The

vector must be able to be maintained stably in the cell over successive generations and can optionally contain particular signals specifying the secretion of the translated enzyme. These various control signals are  
5 chosen as a function of the host cell used. To this end, the nucleic acid sequences may be inserted into autonomous replication vectors within the chosen host or into integrative vectors which become integrated into the genome of the chosen host. Such vectors are  
10 prepared according to the methods commonly used by those skilled in the art, and the clones resulting therefrom may be introduced into a suitable host cell by standard methods such as, for example, heat shock or electroporation.

15 The invention is also directed toward the above cells, characterized in that they are transformed by at least one recombinant isolated nucleic acid encoding the enzyme according to the invention or by at least one  
20 recombinant vector as defined above.

The method according to the invention is characterized in that said modification made by said enzyme is chosen from glycosylation, sulfatation, acetylation,  
25 phosphorylation, succinylation, methylation and addition of an enolpyruvate group, sialylation and fucosylation. More particularly, the method according to the invention is characterized in that said enzyme is an enzyme capable of carrying out a glycosylation,  
30 which is chosen from glycosyl-transferases, glycosyl-hydrolases and glycosyl-phosphorylases. According to one preferred embodiment, the enzyme capable of carrying out the glycosylation is a glycosyl-transferase. According to one preferred embodiment, the  
35 glycosyl-transferase according to the invention is chosen from  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase,  $\beta$ -1,3-galactosyl-transferase,  $\alpha$ -1,3-N-acetyl-galactosaminyl-transferase,  $\beta$ -1,3-glucuronosyl-transferase,  $\beta$ -1,3-N-acetyl-galactosaminyl-transferase,  $\beta$ -1,4-N-

acetyl-galactosaminyl-transferase,  $\beta$ -1,4-galactosyl-transferase,  $\alpha$ -1,3-galactosyl-transferase,  $\alpha$ -1,4-galactosyl-transferase,  $\alpha$ -2,3-sialyl-transferase,  $\alpha$ -2,6-sialyl-transferase,  $\alpha$ -2,8-sialyl-transferase,  $\alpha$ -1,3-fucosyl-transferase,  $\alpha$ -1,4-fucosyl-transferase and  $\alpha$ -1,2-fucosyl-transferase. The glycosyl-transferases used in the present invention are capable of stereospecific conjugation of specific activated saccharide units on a specific acceptor molecule. The activated saccharides generally consist of uridine diphosphate, guanosine diphosphate and cytidine diphosphate saccharide derivatives. Thus, the activated saccharides may be a UDP-saccharide, a GDP-saccharide or a CMP-saccharide.

Certain genes encoding glycosyl-transferases used in the method according to the invention have been described previously; thus, international patent application WO 96/10086 describes the standard oligosaccharide synthesis: in a first step, the various glycosyl-transferases are produced in recombinant bacteria containing the *lgtA*, *lgtB* and *lgtC* genes of *Neisseria gonorrhoeae*, and, after purifying the recombinant enzymes thus produced, the oligosaccharides are synthesized *in vitro* in the presence of the required precursors and sugar-nucleotides.

According to certain embodiments of the invention, the enzyme capable of performing an acetylation is encoded by the *NodL* gene of the bacterium *Azorhizobium caulinodans*. According to another embodiment, the enzyme capable of performing a sulfatation is encoded by the *NodH* gene of the bacterium *Rhizobium meliloti*.

The method according to the invention is characterized in that said cell culturing is preferably performed on a carbon-based substrate; according to one particular embodiment of the invention, said carbon-based substrate is chosen from glycerol and glucose. Other

carbon-based substrates may also be used; mention should be made of maltose, starch, cellulose, pectin and chitin. According to another embodiment, the cell culturing is performed on a substrate composed of amino acids and/or protein and/or lipids.

The method according to the invention is characterized in that said culturing step is performed under conditions allowing the production of a culture with a high cell density; this culturing step comprises a first phase of exponential cell growth ensured by said carbon-based substrate, a second phase of cell growth limited by said carbon-based substrate which is added continuously, and finally a third phase of slowed cell growth obtained by continuously adding to the culture an amount of said substrate that is less than the amount of substrate added in step b) so as to increase the content of oligosaccharides produced in the high cell density culture.

The method according to the invention is characterized in that the amount of substrate added continuously to the cell culture during said phase c) is at least 30% less, preferentially 50% and preferably 60% less than the amount of substrate added continuously during said phase b). The method according to the invention is also characterized in that said exogenous precursor is added during phase b).

According to one embodiment of the invention, the method is characterized in that said exogenous precursor is of carbohydrate nature, preferably of oligosaccharide nature.

The novelty and feasibility of the method according to the invention is based on the use of two modes of internalization of the exogenous precursor that do not destroy the integrity of the cell or attack its vital functions. This especially excludes the standard

techniques of membrane permeabilization with organic solvents which block growth and energy metabolism. The two possible modes for internalizing the exogenous precursor use a passive or active transport mechanism.

5 The invention relates firstly to a method that is characterized in that said exogenous precursor is internalized according to a passive transport mechanism. The expression "internalization by passive  
10 transport" is intended to denote the passive diffusion of any of the exogenous precursor across the plasma membrane, the molecular flow being oriented from the zones of highest concentration to the zones of lowest concentration so as to tend finally toward a state of  
15 equilibrium. The internalization by passive transport consists in using an exogenous precursor that is small enough and hydrophobic enough to diffuse passively across the membrane. A monosaccharide precursor whose anomeric position is blocked with an alkyl substitute  
20 constitutes an example of a precursor that may be internalized in this manner. The present invention thus relates to a method that is characterized in that said exogenous precursor is a monosaccharide whose anomeric carbon is linked to an alkyl group; preferably, said  
25 alkyl group is an allyl group. One of the objects of the invention is thus to provide a method for producing oligosaccharides that contain a functionalizable group such as the allyl group and that can consequently be used as precursors for the chemical synthesis of  
30 glycoconjugates (neoglycoprotein or neoglycolipids) or glycopolymers. The reason for this is that the double bond of the allyl group is able to be opened by ozonolysis to form an aldehyde and to allow the oligosaccharide to conjugate onto a protein by  
35 reductive amination (Roy et al., 1997). Another route is the addition of cysteamine (Lee and Lee, 1974, Roy et al., 1997) to the allylic double bond to form an amine end group which may react, for example, with the carboxylic groups of proteins.



According to one particular embodiment, the method according to the invention concerns the production of ( $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-GlcNac-1 $\rightarrow$ O-allyl); the method is characterized in that said cell is a bacterium of *LacZ*<sup>-</sup> genotype, said enzyme is  $\beta$ -1,4-galactosyl-transferase, said substrate is glycerol and said precursor is allyl-N-acetyl- $\beta$ -D-glucosaminide ( $\beta$ -D-GlcNac-1 $\rightarrow$ O-allyl). Finally, according to another particular embodiment, the method according to the invention is characterized in that the double bond of the allyl group of said ( $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-GlcNac-1 $\rightarrow$ O-allyl) is chemically modified by addition, oxidation or ozonolysis reactions.

The present invention also relates to a method that is characterized in that said precursor is internalized according to an active transport mechanism. The expression "internalization by active transport" is intended to denote the ability of cells and preferably of bacteria to selectively admit and concentrate certain exogenous substances or precursors into their cytoplasm. This transport is performed by transporters of protein nature known as permeases, which act as enzymes; permeases are inducible catalysts, that is to say catalysts that are synthesized in the presence of the substrate or the precursor. According to one particular embodiment of the invention, lactose and  $\beta$ -galactosides constitute precursors that are actively transported into the cytoplasm of the bacterium *Escherichia coli* by lactose permease, also known as galactoside permease. The invention thus relates to a method according to the invention that is characterized in that said active transport of said precursor is performed by lactose permease. Lactose permease has fairly broad specificity, which allows it to transport molecules other than lactose.

The reason for this is that it is capable of transporting various natural or synthetic  $\beta$ -galactosides,  $\alpha$ -galactosides and sucrose. One of the objects of the invention is thus to provide, according to a preferred embodiment, a method that is characterized in that said precursor is lactose, which constitutes the base moiety for a great many biologically active oligosaccharides. It is also within the scope of the invention to provide a method that is characterized in that said precursor is chosen from the group composed of: (i) natural or synthetic  $\beta$ -galactosides, preferably from 4-O- $\beta$ -D-galactopyranosyl-D-fructofuranose (lactulose), 3-O- $\beta$ -D-galactopyranosyl-D-arabinose and allyl- $\beta$ -D-galactopyranoside, (ii)  $\alpha$ -galactosides, preferably melibiose and raffinose, and allyl- $\alpha$ -D-galactopyranoside, (iii) sucrose.

The specificity of lactose permease may even be modified by mutation and allow the transport of other compounds such as maltose and cellobiose. All these compounds may thus be used as precursors for the synthesis of oligosaccharides. It is also within the scope of the invention to use as precursors lactose analogs containing a chemically reactive group for a subsequent functionalization of the product; preferably, one of these analogs is allyl- $\beta$ -D-galactopyranoside. It is also within the scope of this invention to use other permeases possibly modified by recombinant DNA techniques to allow the internalization of different types of precursor.

The  $\beta$ -galactosides are normally hydrolyzed in the cytoplasm of the bacterium by the  $\beta$ -galactosidase encoded by the *LacZ* gene. In order to overcome this problem, a *lacZ*<sup>-</sup> bacterial mutant lacking  $\beta$ -galactosidase activity is used when the precursor used is lactose and/or a  $\beta$ -galactoside. One of the objects of the invention is thus also to provide the method according to the invention that is characterized in

that said cell lacks enzymatic activity liable to degrade said precursor or said metabolic intermediates.

According to one particular embodiment, the invention  
5 relates to a method described above that is characterized in that said precursor is sialic acid. In this case, said active transport of said precursor is performed by NanT permease.

10 According to another particular embodiment, the invention relates to a method described above that is characterized in that said precursor is sialic acid and lactose. In this case, said active transport of said precursor is performed by lactose permease and NanT  
15 permease.

In the method according to the invention, said cell may be lacking in enzymatic activity liable to degrade said precursor(s).

20 According to one preferred embodiment, the method is characterized in that said cell has a genotype chosen from *LacZ*<sup>-</sup> and/or *NanA*<sup>-</sup>.

25 According to another aspect of the invention, the method is characterized in that it also comprises the addition of an inducer to said culture medium to induce the expression in said cell of said enzyme and/or of a protein involved in said active transport; according to  
30 one preferred embodiment, the method according to the invention is characterized in that said inducer is isopropyl  $\beta$ -D-thiogalactoside (IPTG) and said protein is lactose permease.

35 The invention makes it possible for the first time to produce complex oligosaccharides in yields of the order of one gram per liter. Depending on its size, the oligosaccharide either accumulates in the bacterial cytoplasm or is secreted into the culture medium. Thus,

according to one preferred embodiment, the method according to the invention is used for the production of the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl)- $\beta$ -D-galactopyranosyl]-D-glucopyranose,  
5 ( $\beta$ -D-GlcNac-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]-D-Glc); it is characterized in that said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype, said enzyme is  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase, said substrate is glycerol, said inducer is isopropyl  $\beta$ -D-thiogalactoside (IPTG) and said  
10 precursor is lactose.

According to a second preferred embodiment, the method according to the invention is used for the production of lacto-N-neo-tetraose and polylactosamine; it is  
15 characterized in that said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype, said enzymes are  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase and  $\beta$ -1,4-galactosyl-transferase, said substrate is glucose, said inducer is isopropyl- $\beta$ -D-thiogalactoside (IPTG) and said precursor  
20 is lactose.

According to a third preferred embodiment, the method according to the invention is used for the production of allyl 3-O-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl)-  
25  $\beta$ -D-galactopyranoside, ( $\beta$ -D-GlcNac-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-1 $\rightarrow$ O-allyl); it is characterized in that said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype, said enzyme is  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase, said substrate is glycerol, said inducer is isopropyl  $\beta$ -D-  
30 thiogalactoside (IPTG) and said precursor is allyl- $\beta$ -D-galactopyranoside.

According to a fourth preferred embodiment, the method according to the invention is used for the production  
35 of analogs of lacto-N-neo-tetraose and of polylactosamines in which the glucose residue is replaced with an allyl group; it is characterized in that said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype, said enzymes are  $\beta$ -1,3-N-acetyl-glucosaminyl-

transferase and  $\beta$ -1,4-galactosyl-transferase, said substrate is glucose, said inducer is isopropyl  $\beta$ -D-thiogalactoside (IPTG) and said precursor is allyl- $\beta$ -D-galactopyranoside.

5

According to a fifth preferred embodiment, the method according to the invention is used for the production of allyl- $\beta$ -D-lactosamine ( $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-GlcNac-1 $\rightarrow$ O-allyl); it is characterized in that said cell is a  
10 bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype, said enzyme is  $\beta$ -1,4-galactosyl-transferase, said substrate is glycerol and said precursor is allyl-N-acetyl  $\beta$ -D-glucosaminide ( $\beta$ -D-GlcNac-[1 $\rightarrow$ O-allyl]).

15 The invention also relates to a method that makes it possible to envisage the production of a large number of different oligosaccharides obtained by glycosylation of lactose. Specifically, besides the genes *lgtA* and *lgtB* which respectively encode  $\beta$ -1,3-N-acetyl-  
20 glucosaminyl-transferase and  $\beta$ -1,4-galactosyl-transferase, several genes of bacterial glycosyl-transferases using lactose as precursor have recently been cloned. These are *lgtC* ( $\beta$ -1,4-galactosyl-transferase) and *Lst* ( $\alpha$ -2,3-sialyl-transferase)  
25 (Gilbert et al., 1997). Using these genes in a method according to the invention makes it possible to produce molecules such as globotriose (P<sup>k</sup> blood antigen) and sialyl-lactose. Moreover coexpression of the *lgtA* and *lgtB* genes with the gene for  $\alpha$ -1,3-fucosyl-transferase  
30 from *Helicobacter pylori* (Martin et al., 1997) according to a method according to the invention makes it possible to obtain Lewis<sup>x</sup> pentasaccharide. The addition of the *Lst* ( $\alpha$ -2,3-sialyl-transferase) gene gives access to the sialyl Lewis<sup>x</sup> hexasaccharide.

35

The method according to the invention also makes it possible to obtain a large number of different oligosaccharides obtained by glycosylation of exogenous

precursors other than lactose and transported by lactose permease or by other permeases.

The method according to the invention makes it possible to obtain a large number of different oligosaccharides obtained by *in vivo* modification (sulfatation, acetylation, phosphorylation, succinylation, methylation, addition of an enolpyruvate group) of precursors. The synthesis of certain oligosaccharides may necessitate the modification of endogenous precursors, in addition to the modification of exogenous precursors. Thus, it may be envisaged to introduce into a K12 *Escherichia coli* bacterium the gene for the enzyme involved in the metabolism of an endogenous precursor to allow the production of certain sugar-nucleotides such as, for example, CMP-sialic acid, UDP-GalNAc or GDP-fucose, which are not normally produced by this bacterial strain, so as to achieve the synthesis of an oligosaccharide of interest. For example, UDP-GalNAc may be produced from UDP-GlcNAc if the epimerase gene is introduced into a cell according to the invention.

Contrary to the enzymatic method for the *in vitro* synthesis of oligosaccharides, which requires the use of very expensive molecules such as ATP, acetyl-CoA, PAPS (adenosine 3'-phosphate 5'-phosphosulfate) or phospho-enolpyruvate, one of the advantages of the present invention lies in the fact that these molecules are naturally recycled into the cell, thus making it possible to reduce the production costs of the oligosaccharides.

Another subject of the invention relates to a method described above for the production of 3'-sialyllactose ( $\alpha$ -NeuAc-[2 $\rightarrow$ 3]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-Glc) or 6'-sialyllactose ( $\alpha$ -NeuAc-[2 $\rightarrow$ 6]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-Glc), characterized in that:

- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup>, *NanA*<sup>-</sup> or *NanT*<sup>+</sup> genotype;
- said enzymes are CMP-NeuAc-synthase and  $\alpha$ -2,3-sialyl-transferase or  $\alpha$ -2,6-sialyl-transferase;
- 5 • said substrate is glycerol;
- said inducer is isopropyl- $\beta$ -D-thiogalactoside (IPTG);
- said precursors are lactose and sialic acid.

10 According to a sixth preferred embodiment which completes the second embodiment described above, the method according to the invention is used for the production of a sialyl derivative of lacto-N-neo-tetraose and of polylactosamine (lacto-N-neo-hexaose,  
15 lacto-N-neo-octaose, lacto-N-neo-decaose), characterized in that it also comprises a said enzyme chosen from  $\alpha$ -2,3-sialyl-transferase and  $\alpha$ -2,6-sialyl-transferase, and in that said cell also has a *NanA*<sup>-</sup>, *NanT*<sup>+</sup> genotype and expresses the CMP-NeuAc-synthase  
20 gene, said acceptors are lactose and sialic acid.

Another subject of the invention relates to a method described above for the production of lacto-N-neo-tetraose,  $\beta$ -D-Gal[1 $\rightarrow$ 4]- $\beta$ -D-GlcNAc[1 $\rightarrow$ 3]- $\beta$ -D-Gal[1 $\rightarrow$ 4]-  
25 ( $\alpha$ -L-Fuc-[1 $\rightarrow$ 3])- $\beta$ -D-Glc,  $\beta$ -D-Gal[1 $\rightarrow$ 4]-( $\alpha$ -L-Fuc-[1 $\rightarrow$ 3])- $\beta$ -D-GlcNAc[1 $\rightarrow$ 3]- $\beta$ -D-Gal[1 $\rightarrow$ 4]-( $\alpha$ -L-Fuc-[1 $\rightarrow$ 3])- $\beta$ -D-Glc,  
 $\beta$ -D-Gal[1 $\rightarrow$ 4]-( $\alpha$ -L-Fuc-[1 $\rightarrow$ 3])- $\beta$ -D-GlcNAc[1 $\rightarrow$ 3]- $\beta$ -D-Gal[1 $\rightarrow$ 4]- $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]- $\beta$ -D-Gal[1 $\rightarrow$ 4]-( $\alpha$ -L-Fuc-[1 $\rightarrow$ 3])- $\beta$ -D-Glc,  
30 characterized in that

- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup>, *WcaJ* genotype and overexpresses *rcaA*;
- said enzymes are  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase,  $\beta$ -1,4-galactosyl-transferase and  $\alpha$ -  
35 1,3-fucosyl-transferase;
- said substrate is glucose;
- said inducer is isopropyl- $\beta$ -D-thiogalactoside (IPTG);
- said precursor is lactose.

According to a seventh preferred embodiment, the method according to the invention is used for the production of 3'-fucosyllactose ( $\beta$ -D-Gal-[1 $\rightarrow$ 4]-( $\alpha$ -L-Fuc-[1 $\rightarrow$ 3]-D-Glc) or 2'-fucosyllactose ( $\beta$ -D-Gal-[1 $\rightarrow$ 2]-( $\alpha$ -L-Fuc-[1 $\rightarrow$ 3]-D-Glc), characterized in that it comprises a said enzyme chosen from  $\alpha$ -1,3-fucosyl-transferase or  $\alpha$ -1,2-fucosyl-transferase, and in that the cell has a *wcaJ<sup>-</sup> lacZ<sup>-</sup>* genotype and overexpresses the *rcaA* gene and in that said precursor is lactose.

According to an eighth preferred embodiment, the method according to the invention is used for the production of a fucosyl derivative of lacto-N-neo-tetraose and of polylactosamine (lacto-N-neo-hexaose, lacto-N-neo-octaose, lacto-N-neo-decaose), characterized in that it also comprises a said enzyme chosen from  $\alpha$ -1,2-fucosyl-transferase and  $\alpha$ -1,3-fucosyl-transferase, and in that said cell also has a *WcaJ<sup>-</sup>* genotype and overexpresses the *RcsA* gene, said acceptor being lactose.

According to a ninth preferred embodiment, the method according to the invention is used for the production of a sialyl and fucosyl derivative of lacto-N-neo-tetraose, lacto-N-neo-decaose), characterized in that it also comprises a said enzyme chosen from  $\alpha$ -2,3-sialyl-transferase and  $\alpha$ -2,6-sialyl-transferase, and also a said enzyme chosen from  $\alpha$ -1,2-fucosyl-transferase and  $\alpha$ -1,3-fucosyl-transferase, and in that said cell also has a *NanA<sup>-</sup>, NanT<sup>+</sup>, WcaJ<sup>-</sup>* genotype and overexpresses the *RcsA* gene and the gene for CMP-NeuAc-synthase, said acceptors are lactose and sialic acid.

The methods of embodiments 1 to 9 mentioned above may be carried out for the production of oligosaccharide analogs in which the glucose residue is replaced with an allyl group, said precursor now being allyl- $\beta$ -D-galactoside rather than lactose.



Another object of the invention is to provide a method for producing oligosaccharides that are labeled with or enriched in radioisotopes; such oligosaccharides are extremely precious for fundamental biological or conformational analysis studies. The invention thus relates to a method for producing an oligosaccharide that is labeled with at least one radioisotope, characterized in that said cell is cultured on said carbon-based substrate labeled with said radioisotope and/or in the presence of a said precursor labeled with said radioisotope. The radioisotopes are preferably chosen from the group composed of:  $^{14}\text{C}$ ,  $^{13}\text{C}$ ,  $^3\text{H}$ ,  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$ .

The invention also relates to an oligosaccharide which may be obtained by a method according to the invention.

According to one particular embodiment, the invention relates to an activated oligosaccharide that may be used for the chemical synthesis of glycoconjugates or glycopolymers that may be obtained by a method as described above, said oligosaccharide being characterized in that the double bond of the allyl group is chemically modified by addition, oxidation or ozonolysis reactions.

The oligosaccharide according to the invention is useful in a wide range of therapeutic and diagnostic applications; it may be used, for example, as an agent for blocking cell surface receptors in the treatment of a host of diseases involving cellular adhesion, or may be used as nutritional supplements, antibacterial agents, anti-metastatic agents and anti-inflammatory agents. The invention thus relates to an oligosaccharide according to the invention as a medicinal product, and especially as a medicinal product intended for selectively preventing the adhesion of biological molecules. The oligosaccharide according to the invention is also used as a medicinal

product intended for treating cancer, inflammation, heart diseases, diabetes, bacterial infections, viral infections and neurological diseases and as a medicinal product intended for grafts. The invention also relates  
5 to a pharmaceutical composition, characterized in that it comprises an oligosaccharide according to the invention and a pharmaceutically acceptable vehicle.

Finally, the invention also relates to the agricultural  
10 and agronomic use of an oligosaccharide according to the invention, especially for the growth and defense of plants. Specifically, oligosaccharides play a predominant role in Rhizobium/leguminous plant symbiosis. Indeed, certain oligosaccharides originating  
15 from the hydrolysis of fungal or plant glycoproteins or walls can act as plant hormones or as elicitors of defense reactions in plants.

The industrial advantage of the method according to the  
20 invention is obvious since it makes it possible for the first time to achieve a production of the order of a kilogram of complex oligosaccharides of biological interest. All the oligosaccharides of biological interest that we envisage synthesizing at the  
25 industrial scale are currently available only at the mg scale and at extremely high costs (up to 1 million FFr per gram); the cost price of these compounds produced by the present microbiological route are infinitely lower.

30 The characteristics and advantages of the present invention will be demonstrated more clearly on reading the examples and figures which follow, the keys to which are represented below.

35

## FIGURES

Figure 1: Principle of the method for producing the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy- $\beta$ -D-gluco-

**pyranosyl)- $\beta$ -D-galactopyranosyl]-D-glucopyranose, ( $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]-D-Glc)**

Lactose ( $\beta$ -D-Gal-[1-4]- $\beta$ -D-Glc) is transported into the  
5 cell by lactose permease (Lac permease). The lactose cannot be hydrolyzed in the cell since the strain is a *LacZ*<sup>-</sup> mutant. Expression of the *lgtA* gene allows the production of the LgtA enzyme which transfers a GlcNAc from UDP-GlcNAc onto a lactose molecule. The  
10 trisaccharide formed ( $\beta$ -D-GlcNAc-[1-3]- $\beta$ -D-Gal-[1-4]-D-Glc) is excreted into the medium.

**Figure 2: High cell density culturing of the control strain JM 109 and of the strain JM 109 (pCWlgtA) containing the glycosyl transferase gene LgtA**  
15

Lactose is added continuously and the residual lactose is determined enzymatically. The concentration of hydrolyzable GlcNAc in the culture medium is measured  
20 colorimetrically after acid hydrolysis. The added lactose represents the total cumulative amount of lactose which was added continuously.

**Figure 3: Mass spectrum in FAB<sup>+</sup> mode of the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl)- $\beta$ -D-galactopyranosyl]-D-glucopyranose, ( $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]-D-Glc) purified from the culture supernatant of the strain JM 109 (*lgtA*)**  
25

The two quasi-molecular ions [M+H]<sup>+</sup> and [M+Na]<sup>+</sup> are  
30 observed at m/z 546 and 568. An ion [M+H]<sup>+</sup> at m/z 442 is also observed, which is due to the presence of  $\beta$ -D-GlcNAc-[1-3]-IPTG. This indicates that the IPTG (isopropyl  $\beta$ -D-thiogalactose) used to induce Lac  
35 permease and LgtA is also glycosylated.

**Figure 4: Proton NMR spectrum of the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl)- $\beta$ -D-**

galactopyranosyl]-D-glucopyranose, ( $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]-  
 $\beta$ -D-Gal-[1 $\rightarrow$ 4]-D-Glc) at 323° K

5 The signal at 1.4 ppm is due to the protons of the  
isopropyl group of the glycosylated IPTG derivative.

Figure 5:  $^{13}\text{C}$  NMR spectrum of the trisaccharide 4-O-[3-  
O-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl)- $\beta$ -D-  
galactopyranosyl]-D-glucopyranose, ( $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]-  
10  $\beta$ -D-Gal-[1 $\rightarrow$ 4]-D-Glc)

Figure 6: Principle of the method for producing lacto-  
N-neo-tetraose ( $\beta$ -D-Gal-[1-4]- $\beta$ -D-GlcNAc-[1-3]- $\beta$ -D-Gal-  
[1-4]- $\beta$ -D-Glc)

15 Lactose ( $\beta$ -D-Gal-[1-4]- $\beta$ -D-Glc) is transported into the  
cell by Lac permease. The lactose cannot be hydrolyzed  
in the cell since the strain is a *LacZ*<sup>-</sup> mutant.  
Expression of the *lgtA* gene allows the production of  
20 the LgtA enzyme which transfers a GlcNAc from UDP-  
GlcNAc onto a lactose molecule. The trisaccharide  
formed is then used as a precursor by LgtB which  
transfers a galactose molecule from UDP-Gal to form  
lacto-N-neo-tetraose ( $\beta$ -D-Gal-[1-4]- $\beta$ -D-GlcNAc-[1-3]- $\beta$ -  
25 D-Gal-[1-4]- $\beta$ -D-Glc).

Figure 7: High cell density culturing of the strain JM  
109 (pCWlgtA, pBBlgtB)

30 Culturing in the presence of lactose at high  
concentration (5 g.l<sup>-1</sup>) and at low concentration  
(1 g.l<sup>-1</sup>).

Figure 8: Separation on Biogel P4 of the  
35 oligosaccharides produced by the strain JM 109  
(pCWlgtA, pBBlgtB) in the presence of lactose at an  
initial concentration of 5 g.l<sup>-1</sup> (A) or of 1 g.l<sup>-1</sup> (B)

The peaks 1, 2, 3 and 4 correspond, respectively, to lacto-N-neo-tetraose, lacto-N-neo-hexaose, lacto-N-neo-octaose and lacto-N-neo-decaose.

5 **Figure 9: Principle of the method for producing sialyllactose**

Lactose and sialic acid (NeuAc) are internalized in the cell by lactose permease (*lacY*) and sialic acid  
10 permease (*nanT*). These two compounds are not degraded in the cell since the strain is a *lacZ*<sup>-</sup> and *nanA*<sup>-</sup> mutant. The expression of CMP-NeuA synthase and of  $\alpha$ -2,3-sialyl-transferase allows the activation of the sialic acid internalized into CMP-NeuAc and its  
15 transfer onto the intracellular lactose.

**EXAMPLES**

**Example 1: Materials and methods**

20

1.1. Origin of the plasmids and bacterial strains

The strains JM 107 and JM 109 of *Escherichia coli* K12 (Yannisch-Perron et al., 1984) were used as host cells  
25 for all the oligosaccharide production examples described. The strains were obtained from the DSM (Deutsche Sammlung von Mikroorganismen). The genotype of the strain JM 109 is as follows: *F traD36 lacI*<sup>q</sup>  $\Delta(lacZ)M15$  *proA*<sup>+</sup>*B*<sup>+</sup>/*e14*<sup>-</sup>(*McrA*<sup>-</sup>)  $\Delta(lac-proAB)$  *supE44* *recA1*  
30 *endA1* *gyrA96* (*Nal*<sup>r</sup>) *thi* *hsdR17* *relA1*. The genotype of the strain JM 107 is identical to that of the strain JM 109 except for the fact that the *recA1* gene is not inactivated.

35 The *lgtA* and *lgtB* genes of *Neisseria meningitis* MC58 were supplied by Dr W. Wakarchuk (Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa, Ontario, K1A 0R6, Canada) in the form of two plasmids pCW, one containing

the *lgtA* gene (referred to herein as pCWlgtA) and the other containing the *lgtB* gene (referred to herein as pCWlgtB). The sequences of these two genes are available from the GenBank databank under the number  
5 U25839. The plasmid pLitmus28 was purchased from the company New England's Biolabs. The plasmid pBBR1MCS was supplied by Dr M. Kovach (Department of Microbiology and Immunology, Louisiana State University, Shreveport, LA 71130-3932, USA).

10 The genes for CMP-sialic acid synthase and  $\alpha$ -2,3-sialyl-transferase of *Neisseria meningitis* MC58 were supplied by Dr M. Gilbert (Institute for Biological Sciences, National Research Council of Canada, '100  
15 Sussex Drive, Ottawa, Ontario, K1A 0R6, Canada) in the form of two plasmids NSY-01 and NST-01. The plasmid NSY-01 is a derivative of the plasmid pT7-7 which contains the gene (GenBank U60146) for CMP-sialic acid synthase (Gilbert et al., 1997). The plasmid NST-01 is  
20 a derivative of the plasmid pBluescript Sk<sup>-</sup> which contains the gene (GenBank No. U60660) for  $\alpha$ -2,3-sialyl-transferase (Gilbert et al., 1996).

The gene *fucT* for  $\alpha$ -1,3-fucosyl-transferase of  
25 *Helicobacter pylori* was supplied by Dr S. Martin (Glaxo Wellcome Research and Development, Gunnels Wood Road, Stevenage, Hertfordshire, SG1 2NY, UK) in the form of a plasmid pHP0651 derived from pET-21a. The sequence is available from the GenBank (AE000578, gene HP0651).

### 30 1.2. Subclonings

We used the standard techniques of molecular biology described by Sambrook et al. (1989).

35 **construction of the plasmid pBB1gtB:** the 0.835-kb DNA fragment containing the *lgtB* gene was obtained by digestion of the plasmid pCWlgtB with *Bam*HI and *Hind*III. This fragment was subcloned into the vector pLitmus28 predigested with *Bam*HI and *Hind*III to form

the plasmid pLitlgtB. The 0.9-kb fragment containing the *lgtB* gene was excised from the plasmid pLitlgtB by digestion with *XhoI* and *HindIII* and subcloned into the plasmid pBBR1MCS predigested with *XhoI* and *HindIII* to form the plasmid pBBlgtB.

**construction of the plasmid pBBnsy:** The fragment containing the gene for CMP-sialic acid synthase was excised from the plasmid NSY-01 by digestion with *XbaI* and subcloned into the plasmid pBBR1MCS predigested with *XbaI* to form the plasmid pBBnsy.

**construction of the plasmid pBBLnt:** the *lgtA* gene present in the construct pCwlgtA (Gilbert et al.) was amplified by PCR at the same time as the UV5 tactac promoter of the plasmid using the primers CTTTAAGCTTCCGGCTCGTATAA (sense, upstream promoter) and GACAGCTTATCATCGATAAGCTT (antisense, *lgtA* end) both containing a *HindIII* site. The 1.3-kb amplified fragment was then subcloned into the *HindIII* site of the vector pBBlgtB.

**construction of the plasmid pBBLntRcsA:** The *rcaA* gene (Stout et al., 1991) was first amplified by PCR starting with genomic DNA from JM 109 with the primers AGGGTACCCATGTTGTTCCGTTTAG (*KpnI* site, *rcaA* left) and AATCTAGAGTAATCTTATTCAGCCTG (*XbaI* site, *rcaA* right), and then cloned into the *KpnI*-*XbaI* sites of the vector pBBR1-MCS. The vector pBBR1-MCS-*rcaA* was then opened upstream of the gene by digestion with *KpnI*, rendered blunt (Amersham kit), freed with *XbaI* and inserted into the *SmaI*-*XbaI* sites of the construct pBBLnt, allowing a cloning downstream of the *lgtB*-UV5tactac-*lgtA* assembly, placing *rcaA* under the control of the UV5 tactac promoter.

### 1.3. Culture conditions

The routine cultures and the preparation of the inocula were performed on LB medium (Sambrook et al., 1989). The high cell density cultures were prepared in a 2-liter fermenter containing an initial volume of 1 liter of medium having the following composition: glycerol (17.5 g.l<sup>-1</sup>) or glucose (15 g.l<sup>-1</sup>), NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> (7 g.l<sup>-1</sup>), KH<sub>2</sub>PO<sub>4</sub> (7 g.l<sup>-1</sup>), MgSO<sub>4</sub>·7H<sub>2</sub>O (1 g.l<sup>-1</sup>), thiamine HCl (4.5 mg.l<sup>-1</sup>), solution of trace elements (7.5 ml.l<sup>-1</sup>), citric acid (0.5 g.l<sup>-1</sup>), KOH (2 g.l<sup>-1</sup>). The MgSO<sub>4</sub> is autoclaved separately and the thiamine is sterilized by filtration. The solution of trace elements contains: nitrilotriacetate (70 mM, pH 6.5), ferric citrate (7.5 g.l<sup>-1</sup>), MnCl<sub>2</sub>·4H<sub>2</sub>O (1.3 g.l<sup>-1</sup>), CoCl<sub>2</sub>·6H<sub>2</sub>O (0.21 g.l<sup>-1</sup>), CuCl<sub>2</sub>·2H<sub>2</sub>O (0.13 g.l<sup>-1</sup>), H<sub>3</sub>BO<sub>3</sub> (0.25 g.l<sup>-1</sup>), ZnSO<sub>4</sub>·7H<sub>2</sub>O (1.2 g.l<sup>-1</sup>), Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (0.15 g.l<sup>-1</sup>). The antibiotics ampicillin (50 mg.l<sup>-1</sup>) and chloramphenicol (25 mg.l<sup>-1</sup>) are added to ensure the presence of the various plasmids. The feed solution contains glycerol (500 g.l<sup>-1</sup>) or glucose (400 g.l<sup>-1</sup>), MgSO<sub>4</sub>·7H<sub>2</sub>O (12 g.l<sup>-1</sup>) and the solution of trace elements (25 ml.l<sup>-1</sup>).

The high cell density cultures are inoculated at 2%. Throughout the culturing, the dissolved oxygen content is maintained at 20% of saturation by manually controlling the flow rate of air and by automatically adjusting the stirring speed. The pH is automatically maintained at 6.8 by addition of aqueous ammonia (15% w/v). The temperature is maintained at 34°C for the strain JM 109(pCWlgtA) and at 28°C for the strain JM 109(pCWlgtA, pBBlgtB). The high-density culture strategy generally comprises three phases: a first phase of exponential growth which is ensured by the carbon-based substrate (glycerol or glucose) initially present in the medium; a second phase which starts when the growth becomes limited by the carbon source, which is then added continuously at a rate of 4.5 g.h<sup>-1</sup>.l<sup>-1</sup> of glycerol or 3.6 g.h<sup>-1</sup>.l<sup>-1</sup> of glucose. In a third phase, this rate is reduced by 60% to slow down the growth, so as to increase the oligosaccharide content.



#### 1.4. Assay of the oligosaccharides

Samples (1 ml) are taken during the culturing and are  
5 immediately centrifuged in microtubes. The supernatant  
is retained to assay the extracellular oligosacchar-  
ides. The bacterial pellet is resuspended in 1 ml of  
water and is then incubated in a water bath at 100°C  
for 30 minutes to rupture the cells. After a second  
10 centrifugation, the supernatant is retained to assay  
the intracellular oligosaccharides.

The lactose concentration is measured using an  
enzymatic determination kit (Roche diagnostic). The N-  
15 acetyl-glucosamine residues present in the  
oligosaccharides are freed by acid hydrolysis as  
described previously (Samain et al., 1997) and then  
quantified colorimetrically by the method of Reissig et  
al., (1955); in the description, the term "hydrolyzable  
20 GlcNAc" means the amount of GlcNAc assayed in this way.

Assaying the lactose with and without treatment with a  
neuraminidase makes it possible to estimate the sialyl-  
lactose concentration.

25 The total fucose is measured colorimetrically by the  
cysteine hydrochloride method of Dische and Shettles  
(1948).

#### 30 1.5. Purification of the oligosaccharides

At the end of the culturing, the bacterial cells are  
harvested by centrifugation. The supernatant is  
retained for purification of the extracellular  
35 oligosaccharides. The bacterial cells are resuspended  
in 1 liter of water and are then permeabilized by means  
of a heat treatment (30 minutes at 100°C) to release  
the intracellular oligosaccharides. After a second

centrifugation, these oligosaccharides are recovered in the supernatant.

5 The first and the second supernatant containing the extracellular and intracellular oligosaccharides, respectively, are adsorbed onto active charcoal (100 g per liter of supernatant). After rinsing with distilled water, the oligosaccharides are diluted with 50% (v/v) ethanol, concentrated by evaporation and freeze-dried.

10 The oligosaccharides are separated out by steric exclusion chromatography on a column (4.5 cm x 95 cm) of Biogel P4, allowing the injection of about 300 mg of oligosaccharide mixture. The elution is performed with  
15 distilled water, at a flow rate of 40 ml.h<sup>-1</sup>.

The nonfucosyl oligosaccharides are separated out by steric exclusion chromatography on a column (4.5 cm x 95 cm) of Biogel P4, allowing the injection of about  
20 300 mg of oligosaccharide mixture. The elution is performed with distilled water at a flow rate of 40 ml.h<sup>-1</sup>.

The fucosyl oligosaccharides are separated out by  
25 steric exclusion chromatography on a column (1.5 cm x 200 cm) of Biogel P2 thermostatically maintained at 60°C, allowing the injection of about 30 mg of oligosaccharide mixture. The elution is performed with distilled water at a flow rate of 30 ml.h<sup>-1</sup>.

30 The sialyllactose is separated from the neutral oligosaccharides by binding onto a Dowex 1X4-400 resin (in HCO<sub>3</sub><sup>-</sup> form), and eluted with a gradient of NaHCO<sub>3</sub> (0 to 100 mM). The bicarbonate is then removed by treating  
35 the eluate with a Dowex 50X4-400 resin in H<sup>+</sup> form.

#### 1.6. Preparation of the allyl β-D-glucosides

Allyl  $\beta$ -D-galactopyranoside and allyl-N-acetyl- $\beta$ -D-glucosaminide were synthesized according to the protocol described by Lee and Lee (1974).

5 1.7. Identification and structural characterization of the oligosaccharides

The mass spectra were acquired using a mass spectrometer (Nermag R-1010C). For each experiment, the  
10 initial matrix volume is 4  $\mu$ l. The products were analyzed in FAB<sup>+</sup> mode.

The NMR spectra were obtained using a Bruker AC300 spectrometer.

15

1.8. Construction of the strain JM 107-nanA-

A JM 107 strain incapable of metabolizing sialic acid was prepared by insertional inactivation of the *nanA* gene (Nan operon) encoding NeuAc aldolase (Plumbridge  
20 et al., 1999). Two PCR amplification reactions were performed on either side of the center of the *nanA* gene so as to insert therein a *Bam*HI restriction site.

25 A first 1.6-kb *Bam*HI-*Xba*I fragment comprising the right-hand portion of *nanA* was amplified from the genomic DNA of JM 109 using the primers AAAGGATCCAAGATCAGGATGTTACG and GCTCTAGAATGGTAATGATGAGGCAC and cloned between the *Bam*HI and *Xba*I sites of the vector pUC19, forming the vector  
30 pUC-nan1.6. A second 2.1-kb *Kpn*I-*Bam*HI fragment comprising the left-hand portion of *nanA* was amplified using the primers AAAGGATCCGCGTAGGTGCGCTGAAAC and AAAGGTACCTCAGGCCACCGTTAGCAG and cloned between the *Kpn*I and *Bam*HI sites of the vector pUC-nan1.6, forming the  
35 vector pUC-nan3.7. The kanamycin-resistance gene (pUC-4K, Pharmacia cassette) was then cloned into the *Bam*HI site of pUC-nan3.7. The 4.9-kb *Sac*I-*Xba*I fragment containing *nanA::kan* was inserted into the same sites

of the suicide vector pCVD442 (Donnenberg and Kaper, 1991). This plasmid was used to obtain by homologous recombination JM 107 *nanA::kan* mutants, selected for their resistance to kanamycin and their inability to metabolize sialic acid (strain JM 107-*nanA*<sup>-</sup>).

#### 1.9. Construction of the strain JM 107col<sup>-</sup>DE3

Suppression of the capacity to synthesize colanic acid was achieved by insertional inactivation of the *wcaJ* gene encoding a glucosyl-transferase (Stevenson et al., 1996). A 1.8-kb DNA fragment containing the *wcaJ* gene and adjacent DNA were amplified by PCR starting with genomic DNA from JM 109, and inserted into a vector pTOPO2.1 (Invitrogen PCR cloning kit), with the aid of the primers CCACGATCCACGTCTCTCC (right *wcaJ*) and AAGCTCATATCAATATGCCGCT (left *wcaJ*). It was then transferred into a pUC19 vector into the *EcoRI* site. The vector thus obtained was subjected to a treatment with an *EcoRI* methylase, allowing the subsequent addition of the kanamycin-resistance gene to the *ApoI* site present at the center of *wcaJ*. The recombinant DNA *wcaJ::kan* was finally transferred into the suicide vector pCVD442 allowing, by homologous recombination, the production of JM 107 genomic mutants containing the inactivated gene, selected by PCR with the aid of the primers which were used for the cloning (strain JM 107-*col*<sup>-</sup>).

The strain JM 107-*col*<sup>-</sup> was made lysogenic for the phage  $\lambda$ DE3 using the lysogenization kit from Novagen.

**Example 2: production of the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl)- $\beta$ -D-galactopyranosyl]-D-glucopyranose, ( $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]-D-Glc)**

The principle is illustrated by Figure 1. We used the strain JM 109 of *Escherichia coli* K12, into which we

introduced the plasmid pCWlgtA *lgtA* gene. The strain JM 109 is *lacZ*<sup>-</sup>, that is to say that it is incapable of hydrolyzing lactose. On the other hand, it is *lacY*<sup>+</sup>, which means that it can synthesize lactose permease.

5 The *lgtA* gene encodes a  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase (LgtA), which transfers an N-acetyl-glucosamine unit onto the galactose of lactose.

The strain JM 109 (pCWlgtA) and also the JM 109 control strain were cultured at high cell density (Samain *et al.*, 1997) on glycerol as the carbon and energy sources. After the first phase of exponential growth provided by the glycerol initially present in the medium (17.5 g/l), the growth becomes limited by the glycerol, which is then added continuously at a rate of 4.5 g.h<sup>-1</sup>.l<sup>-1</sup>. During this second culturing phase, 90 mg.h<sup>-1</sup>.l<sup>-1</sup> of lactose are introduced continuously. IPTG (isopropyl- $\beta$ -D-thiogalactoside) (0.5 mM) is also injected at the start of this phase to induce the expression of the lactose permease and of the  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase. As described in Figure 2, the added lactose is virtually not accumulated in the medium, indicating that the lactose is indeed internalized by the bacterial cells. A large accumulation in the culture medium of a compound containing N-acetylglucosamine (hydrolyzable GlcNAc) is observed with the strain JM 109 (pCWlgtA). The amount of hydrolyzable GlcNAc (3.8 mmol/l) produced corresponds almost stoichiometrically to the amount of lactose consumed (3.5 mmol/l), suggesting that all of the lactose internalized has been glycosylated by LgtA.

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15  
20  
25  
30

At the end of the culturing, the cells are removed by centrifugation and the oligosaccharides present in the supernatant are purified by adsorption onto active charcoal and elution with ethanol. The oligosaccharides present are then separated out according to their molecular weight, on a Biogel P4 column. A single predominant compound is found. The mass spectrometry

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and NMR data indicate that this compound is indeed the trisaccharide ( $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-Glc) formed by the addition of a GlcNAc residue to a lactose molecule. Indeed, the mass spectrum in FAB<sup>+</sup> mode shows the presence of a quasi-molecular ion [M+H]<sup>+</sup> at m/z 546 (Figure 3). The <sup>1</sup>H NMR spectrum confirms the trisaccharide structure, the presence of an acetyl group and the  $\beta$  configuration of the two O-glycoside linkages (Figure 4). The <sup>13</sup>C NMR spectrum also specifies that the linkage between the GlcNAc and the galactose is indeed of 1,3 type (Figure 5).

### **Example 3: Production of lacto-N-neo-tetraose and of polylactosamine**

The principle is described in Figure 6. The strain of *E. coli* JM 109 was cotransformed with the two plasmids pCWlgtA and pBBlgtB bearing, respectively, the genes *lgtA* (used previously) and *lgtB* (encoding a  $\beta$ -1,4-galactosyl-transferase known as LgtB). The strain JM 109 (pCWlgtA, pBBlgtB) was cultured at high cell density using glucose as the growth substrate. At the start of the second phase, lactose is added at high concentration (5 g.l<sup>-1</sup>) or at low concentration (1 g.l<sup>-1</sup>) and 0.1 mM IPTG is added. Contrary to what was observed with the strain JM 109 (pCWlgtA), only a weak release of hydrolyzable GlcNAc into the medium is detected during the culturing of this strain. On the other hand, hydrolyzable GlcNAc is found in large amount in the bacterium (Figure 7). When the supply of lactose is 1 g.l<sup>-1</sup>, complete internalization of the lactose (2.9 mmol.l<sup>-1</sup>) and a total production of bound GlcNAc of 1.45 g.l<sup>-1</sup> (6.5 mmol.l<sup>-1</sup>), i.e. the incorporation of more than 2 GlcNAc molecules per acceptor lactose molecule, are observed. When the lactose is added in high concentration, the internalization is incomplete (3 g.l<sup>-1</sup>, i.e. 8.7 mmol.l<sup>-1</sup>) with a production of GlcNAc also of about 6.5 mmol.l<sup>-1</sup>. In this case, the GlcNAc/lactose molar

ratio is close to 1, which is coherent with the synthesis of lacto-N-neo-tetraose.

The purification of the intracellular oligosaccharide fraction made it possible to obtain several main compounds that are well separated by chromatography on Biogel P4. The mass spectrometry and NMR data indicate that these compounds correspond to the following structures: lacto-N-neo-tetraose  $[M+H]^+ = 708$ ; lacto-N-neo-hexaose  $[M+H]^+ = 708$ ; lacto-N-neo-octaose  $[M+Na]^+ = 1460$  and probably lacto-N-neo-decaose. The respective proportions of these various compounds depend on the amount of lactose added. Thus, with  $5 \text{ g.l}^{-1}$  of lactose, the major product is lacto-N-neo-tetraose (Figure 8A). On the other hand, a lower supply of lactose ( $1 \text{ g.l}^{-1}$ ) promotes the formation of compounds with a higher degree of polymerization, lacto-N-neo-octaose becoming the major product (Figure 8B).

The formation of higher polylactosamine homologs of lacto-N-neo-tetraose is explained by the fact that LgtA is capable of using lacto-N-neo-tetraose to form an intermediate pentasaccharide that is glycosylated by LgtB to give lacto-N-neo-hexaose. The latter compound is itself the precursor for a new glycosylation cycle resulting in the formation of lacto-N-neo-octaose, and so on upto lacto-N-neo-decaose.

No significant formation of oligosaccharides with an odd number of residues and bearing a galactose in a nonreducing end position is observed. This indicates that the elongation of the molecules is limited by the incorporation of GlcNAc by LgtA rather than by the galactosylation catalyzed by LgtB.

35

**Example 4: Production of allyl 3-O-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl)- $\beta$ -D-galactopyranoside, ( $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-1 $\rightarrow$ O-allyl)**

The strain JM 109(pCWlgtA) was cultured at high cell density on glycerol. At the start of the second phase of culturing, 0.75 g.l<sup>-1</sup> of allyl- $\beta$ -D-galactopyranoside and 0.1 mM of IPTG are added. A total internalization  
5 of the allyl- $\beta$ -D-galactopyranoside is observed after 9 hours, with a stoichiometric appearance of hydrolyzable GlcNAc in the extracellular medium. The oligosaccharides present in the extracellular medium are purified as in Example 2. The mass spectrum in FAB<sup>+</sup>  
10 mode of the major product obtained shows the presence of a quasi-molecular ion [M+H]<sup>+</sup> at m/z 424 corresponding to the structure  $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-A $\rightarrow$ O-allyl.

15 **Example 5: Production of  $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-GlcNAc-1 $\rightarrow$ O-allyl**

The strain JM 109 (pBB1gtB) was cultured at high cell density on glycerol. At the start of the second phase  
20 of culturing, 0.5 g.l<sup>-1</sup> of allyl-N-acetyl- $\beta$ -D-glucosaminide ( $\beta$ -D-GlcNAc-1 $\rightarrow$ O-allyl) is added. An approximately 30% reduction in the amount of extracellular hydrolyzable GlcNAc is observed in the first five hours, which demonstrates a partial  
25 internalization of allyl-N-acetyl- $\beta$ -D-glucosaminide. In parallel, an almost stoichiometric intracellular production of hydrolyzable GlcNAc and of  $\beta$ -linked galactose residues (hydrolyzable with  $\beta$ -galactosidase) is observed. These results demonstrate that 30% of the  
30 allyl-N-acetyl- $\beta$ -D-glucosaminide initially added has been galactosylated by the activity encoded by the *lgtB* gene. After purification, the structure of the expected compound ( $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-GlcNAc-1 $\rightarrow$ O-allyl) was confirmed by mass spectrometry and NMR.

35

**Example 6: Production of analogs of lacto-N-neotetraose and of polylactosamines in which the glucose residue is replaced with an allyl group**



The strain JM 109 (pCWlgtA and pBBlgtB) was cultured as in Example 3, except that the supply of lactose was replaced with the addition of 0.65 g.l<sup>-1</sup> of allyl- $\beta$ -D-galactopyranoside. After purification according to the  
5 protocol of Example 3, three main compounds are obtained. The mass spectrometry data indicate that these three compounds correspond to the tri-, penta- and heptasaccharides below:

$\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-1 $\rightarrow$ O-allyl,

10 [M+H] = 586;

$\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-1 $\rightarrow$ O-allyl, [M+H] = 951;

$\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-GlcNAc-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-1 $\rightarrow$ O-allyl, [M+H] = 1316.  
15

**Example 7: Production of 3'-sialyllactose ( $\alpha$ -NeuAc-[2 $\rightarrow$ 3]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-Glc)**

20 The principle is illustrated by Figure 9. The genes for the biosynthesis of sialic acid and CMP-NeuAc are not present in *E. coli* K12. However, *E. coli* K12 is capable of degrading sialic acid (Plumbridge and Vimr, 1999) and contains a permease (NanT) that enables exogenous  
25 sialic acid to enter into the cell. This sialic acid is then normally catabolized by an aldolase (NanA).

We used the strain of *Escherichia coli* K12 JM 107-nanA<sup>-</sup> (Example 1) and a JM 107 control strain into which we  
30 introduced the two compatible plasmids NST-01 and pBBnsy containing, respectively, the genes for  $\alpha$ -2,3-sialyl-transferase and for CMP-NeuAc synthase. This strain lacks nanA activity and is thus incapable of degrading intracellular sialic acid. However, it  
35 contains the lactose permease (*lacY*) and sialyl permease (*nanT*) genes and is thus capable of internalizing exogenous sialic acid and lactose. The internalized sialic acid may thus be activated to CMP-NeuAc by the action of CMP-NeuAc synthase and

transferred onto the intracellular lactose by the action of  $\alpha$ -2,3-sialyl-transferase.

The strain JM 107-nanA<sup>-</sup>(Nst-01, pBBnsy) and the JM 107 control strain (Nst-01, pBBnsy) having the NanA activity were cultured at high cell density on glycerol. Lactose (1.5 g.l<sup>-1</sup>), IPTG (0.1 mM) and sialic acid (0.6 g.l<sup>-1</sup>) are added at the start of the second phase of culturing for a period of five hours. Throughout the duration (17 hours) of the third phase of culturing, 100 mg.h<sup>-1</sup>.L<sup>-1</sup> of sialic acid and 200 mg.h<sup>-1</sup>.L<sup>-1</sup> of lactose are introduced continuously.

At the end of culturing of the strain JM 107-nanA<sup>-</sup>(Nst-01, pBBnsy), enzymatic assay of the lactose with and without treatment with a neuraminidase makes it possible to estimate the total production of sialyllactose as 2.6 g.l<sup>-1</sup>. This production is partly found in the bacterial cells (1.5 g.l<sup>-1</sup>) and in the extracellular culture medium (1.1 g.l<sup>-1</sup>). In the case of the JM 107 control strain (Nst-01, pBBnsy), the production of sialyllactose is much lower (150 mg.l<sup>-1</sup>), indicating that virtually all of the sialic acid has been degraded by the bacterium.

The intracellular and extracellular oligosaccharides are purified by adsorption onto active charcoal and elution with ethanol. After purification on an anion-exchange resin, only one product is detected by HPLC. The mass spectrum in FAB<sup>+</sup> mode shows the presence of two quasi-molecular ions [M+H]<sup>+</sup> at m/z 656 and [M+Na] at m/z 678 corresponding to the sodium salt of sialyllactose.

#### **Example 8: Production of fucosyl derivatives of lacto-N-neo-tetraose**

In *E. coli* K12, the genes for the biosynthesis of GDP-fucose form part of the operon responsible for the

biosynthesis of an extracellular polysaccharide, colanic acid (Stevenson et al., 1996). The expression of this operon is controlled by a complex regulation network in which the protein RcsA is involved (Stout et al., 1991). The overexpression of the *rcsA* gene is thus reflected by an overproduction of colanic acid (Russo and Singh, 1993) and consequently of the genes for the biosynthesis of GDP fucose.

10 To increase the availability of GDP fucose, our strategy consisted in using a strain of *E. coli* in which the *rcsA* gene was overexpressed (so as to overproduce the genes for the biosynthesis of GDP-fucose) and in which one of the genes that is essential  
15 to the biosynthesis of colanic acid has been inactivated (so as to totally suppress the production of colanic acid and to avoid a competition for the use of GDP-fucose).

20 We used the strain JM 107-col<sup>-</sup>DE3 in which the *wcaJ* gene, which is responsible for the transfer of the first glucose residue of the repeating unit, has been inactivated according to Example 1 and into which we introduced either the two plasmids pHP0651 and pBBLnt,  
25 or the two plasmids pHP0651 and pBBLntRcsA. The plasmid pHP0651 contains the *fucT* gene for  $\alpha$ -1,3-fucosyl-transferase of *Helicobacter pylori*. This fucosyl-transferase uses as acceptor N-acetyllactosamine and lacto-N-neo-tetraose, but not lactose (Martin et al.,  
30 1997). The plasmid pBBLnt contains the *lgtA* and *lgtB* genes. The plasmid pBBLntRcsA contains the *lgtA*, *lgtB* and *rcsA* genes.

The two strains JM 107-col<sup>-</sup>DE3 (pHP0651, pBBLnt) and JM  
35 107-col<sup>-</sup>DE3 (pHP0651, pBBLntRcsA) were cultured as in Example 3 in the presence of 5 g.l<sup>-1</sup> of lactose. At the end of the third phase of culturing, the amount of hydrolyzable GlcNAc produced by the two strains (1.7 g.l<sup>-1</sup>) was comparable to that obtained with the

strain JM 109 (pCWlgtA, pBBlgtB) in Example 3. The colorimetry assay of the fucose at the end of culturing shows a large difference between the two strains, with a fucose production of  $1 \text{ g.l}^{-1}$  for the strain JM 107-  
5 col<sup>-</sup>DE3 (pHP0651, pBBLntRcsA) and only  $0.25 \text{ g.l}^{-1}$  for the strain JM 107-col<sup>-</sup>DE3 (pHP0651, pBBLnt). The fucosyl oligosaccharides are found at more than 70% in the intracellular fraction.

10 Purification of the intracellular fraction by adsorption onto active charcoal and steric exclusion chromatography on Biogel P2 makes it possible to separate four main compounds.

15 Compound 1 corresponds, by its elution volume on Biogel P2 and its thin-layer migration, to lacto-N-neo-tetraose.

The mass spectrum of compound 2 shows the presence of a  
20 quasi-molecular ion  $[M+H]^+$  at  $m/z$  854 corresponding to the molar mass of lacto-N-fucopentaose. The presence of a secondary ion at 327 indicates that the molecule is fucosylated on the glucose residue and has the following structure

25  $\beta\text{-D-Gal-[1}\rightarrow\text{4]}\text{-}\beta\text{-D-GlcNAc-[1}\rightarrow\text{3]}\text{-}\beta\text{-D-Gal-[1}\rightarrow\text{4]}\text{-(}\alpha\text{-L-Fuc-[1}\rightarrow\text{3])}\text{-}\beta\text{-D-Glc.}$

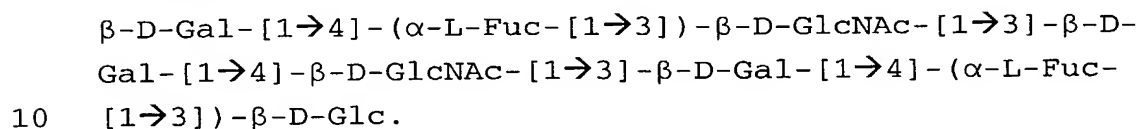
The mass spectrum of the major compound 3 shows the presence of 3 quasi-molecular ions at  $m/z$  1000, 1022  
30 and 1038 corresponding to the three forms  $[M+H]^+$ ,  $[M+Na]^+$  and  $[M+K]^+$  of the lacto-N-difucohexaose molecule having the following structure

$\beta\text{-D-Gal-[1}\rightarrow\text{4]}\text{-(}\beta\text{-L-Fuc-[1}\rightarrow\text{3])}\text{-}\beta\text{-D-GlcNAc-[1}\rightarrow\text{3]}\text{-}\beta\text{-D-Gal-[1}\rightarrow\text{4]}\text{-(}\alpha\text{-L-Fuc-[1}\rightarrow\text{3])}\text{-}\beta\text{-D-Glc.}$

35

The mass spectrum of compound 4 makes it possible to identify two quasi-molecular ions at  $m/z$  1365 and 1388 corresponding to the  $[M+H]^+$  and  $[M+Na]^+$  forms of a lacto-N-difucooctaose molecule. The presence of a

secondary ion at m/z 512 indicates that the GlcNAc residue of the nonreducing end bears a fucose. The NMR data show that the <sup>1</sup>H proton of a fucose residue is sensitive to anomerism and that this fucose residue is thus bound to the glucose. These results make it possible to propose the following structure for compound 4:



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CLAIMS

1. A method for producing an oligosaccharide of interest by a genetically modified cell starting with at least one internalized exogenous precursor, said precursor being involved in the biosynthetic pathway of said oligosaccharide, said method comprising the steps of:
- (i) obtaining a cell that
- comprises at least one recombinant gene encoding an enzyme capable of modifying said exogenous precursor or one of the intermediates in the biosynthetic pathway of said oligosaccharide from said exogenous precursor necessary for the synthesis of said oligosaccharide from said precursor, and also the components for expressing said gene in said cell,
  - lacks any enzymatic activity liable to degrade said oligosaccharide, said precursor and said intermediates;
- (ii) culturing said cell in the presence of at least one said exogenous precursor, under conditions enabling the internalization according to a mechanism of passive and/or active transport of said exogenous precursor by said cell and the production of said oligosaccharide by said cell.
2. The method as claimed in claim 1, characterized in that said cell also comprises at least one gene encoding an enzyme capable of modifying an endogenous precursor involved in the biosynthetic pathway of said oligosaccharide, said enzyme being identical to or different than the enzyme used in the method described above, and also to the components for expressing said gene in said cell and characterized in that said cell lacks any



enzymatic activity liable to degrade said precursor.

3. The method as claimed in claim 1 or 2,  
5 characterized in that said cell is a cell chosen from bacteria and yeasts.
4. The method as claimed in claim 3, characterized in  
10 that the cell is a bacterium, preferably of *Escherichia coli* type.
5. The method as claimed in one of claims 1 to 4,  
15 characterized in that said modification is chosen from glycosylation, sulfatation, acetylation, phosphorylation, succinylation, methylation, and addition of an enolpyruvate group.
6. The method as claimed in one of claims 1 to 5,  
20 characterized in that said enzyme is an enzyme capable of performing a glycosylation, chosen from glycosyl-transferases.
7. The method as claimed in claim 6, characterized in  
25 that said enzyme is a glycosyl-transferase chosen from  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase,  $\beta$ -1,3-galactosyl-transferase,  $\alpha$ -1,3-N-acetyl-galactosaminyl-transferase,  $\beta$ -1,3-glucuronosyl-transferase,  $\beta$ -1,3-N-acetyl-galactosaminyl-transferase,  $\beta$ -1,4-N-acetyl-galactosaminyl-transferase,  $\beta$ -1,4-galactosyl-transferase,  $\alpha$ -1,3-galactosyl-transferase,  $\alpha$ -1,4-galactosyl-transferase,  $\alpha$ -2,3-sialyl-transferase,  $\alpha$ -2,6-sialyl-transferase,  $\alpha$ -2,8-sialyl-transferase,  $\alpha$ -1,2-fucosyl-transferase,  $\alpha$ -1,3-fucosyl-transferase  
30 and  $\alpha$ -1,4-fucosyl-transferase.  
35

8. The method as claimed in any one of claims 1 to 7, characterized in that said cell culturing is carried out on a carbon-based substrate.
- 5 9. The method as claimed in claim 8, characterized in that said carbon-based substrate is chosen from glycerol and glucose.
- 10 10. The method as claimed in one of claims 8 and 9, characterized in that said culturing is performed under conditions allowing the production of a culture with a high cell density.
- 15 11. The method as claimed in claim 10, characterized in that said culturing step comprises:
  - a)- a first phase of exponential cell growth ensured by said carbon-based substrate,
  - b)- a second phase of cell growth limited by said carbon-based substrate which is added
  - 20 continuously,
  - c)- a third phase of slowed cell growth obtained by continuously adding to the culture an amount of said substrate that is less than the amount of substrate added in step b) so as to increase the
  - 25 content of oligosaccharides produced in the high cell density culture.
- 30 12. The method as claimed in claim 11, characterized in that the amount of substrate added continuously to the cell culture during said phase c) is at least 30% less, preferentially 50% and preferably 60% less than the amount of substrate added continuously during said phase b).
- 35 13. The method as claimed in either of claims 11 and 12, characterized in that said precursor is added during phase b).

14. The method as claimed in one of claims 1 to 13, characterized in that said precursor is of carbohydrate nature, preferably of oligosaccharide nature.
- 5
15. The method as claimed in claim 1, characterized in that said precursor is a monosaccharide whose anomeric carbon is linked to an alkyl group so as to allow its internalization by a mechanism of
- 10 passive transport.
16. The method as claimed in claim 15, characterized in that said alkyl group is an allyl.
- 15
17. The method as claimed in either of claims 15 and 16, for the production of ( $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-GlcNac-1 $\rightarrow$ O-allyl), characterized in that
- said cell is a bacterium of *LacZ*<sup>-</sup> genotype;
  - said enzyme is  $\beta$ -1,4-galactosyl-transferase;
  - 20 • said substrate is glycerol;
  - said precursor is allyl-N-acetyl- $\beta$ -D-glucosaminide ( $\beta$ -D-GlcNac-1 $\rightarrow$ O-allyl).
18. The method as claimed in claim 1, characterized in
- 25 that said precursor is lactose.
19. The method as claimed in claim 1, characterized in that said precursor is chosen from the group composed
- 30
- of natural or synthetic  $\beta$ -galactosides, preferably from 4-O- $\beta$ -D-galactopyranosyl-D-fructofuranose (lactulose), 3-O- $\beta$ -D-galactopyranosyl-D-arabinose and allyl- $\beta$ -D-galactopyranoside;
  - 35 • of  $\alpha$ -galactosides, preferably melibiose and raffinose, and allyl- $\alpha$ -D-galactopyranoside,
  - of sucrose.

20. The method as claimed in either of claims 18 and 19, characterized in that said active transport of said precursor is performed by lactose permease.
- 5 21. The method as claimed in claim 1, characterized in that said precursor is sialic acid.
22. The method as claimed in claim 21, characterized in that said active transport of said precursor is performed by NanT permease.
- 10 23. The method as claimed in claim 1, characterized in that said precursor is sialic acid and lactose.
- 15 24. The method as claimed in claim 23, characterized in that said active transport of said precursor is performed by lactose permease and NanT permease.
- 20 25. The method as claimed in any one of claims 1 to 24, characterized in that said cell lacks any enzymatic activity liable to degrade said precursor(s).
- 25 26. The method as claimed in claim 25, characterized in that said cell has a genotype chosen from *LacZ*<sup>-</sup> and/or *NanA*<sup>-</sup>.
- 30 27. The method as claimed in any one of claims 1 to 26, characterized in that it also comprises the addition of an inducer to said culture medium to induce the expression in said cell of said enzyme and/or of a protein involved in said active transport.
- 35 28. The method as claimed in claim 27, characterized in that said inducer is isopropyl  $\beta$ -D-thiogalactoside (IPTG) and said protein is lactose permease.

29. The method as claimed in any one of claims 1 to 28, for the production of the trisaccharide 4-O-[3-O-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl)- $\beta$ -D-galactopyranosyl]-D-glucopyranose, ( $\beta$ -D-GlcNac-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]-D-Glc), characterized in that:
- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype;
  - said enzyme is  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase;
  - said substrate is glycerol;
  - said inducer is isopropyl  $\beta$ -D-thiogalactoside (IPTG);
  - said precursor is lactose.
30. The method as claimed in any one of claims 1 to 28, for the production of lacto-N-neo-tetraose and polylactosamine (lacto-N-neo-hexaose, lacto-N-neo-octaose, lacto-N-neo-decaose), characterized in that:
- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup> genotype;
  - said enzymes are  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase and  $\beta$ -1,4-galactosyl-transferase;
  - said substrate is glucose;
  - said inducer is isopropyl- $\beta$ -D-thiogalactoside (IPTG);
  - said precursor is lactose.
31. The method as claimed in claim 30, for the production of a sialyl derivative of lacto-N-neo-tetraose and of polylactosamine (lacto-N-neo-hexaose, lacto-N-neo-octaose, lacto-N-neo-decaose), characterized in that it also comprises a said enzyme chosen from  $\alpha$ -2,3-sialyl-transferase and  $\alpha$ -2,6-sialyl-transferase, and in that said

cell also has a *NanA*<sup>-</sup>, *NanT*<sup>+</sup> genotype and expresses the gene for CMP-NeuAc-synthase.

32. The method as claimed in claim 30, for the  
5 production of a fucosyl derivative of lacto-N-neo-  
tetraose and of polylactosamine (lacto-N-neo-  
hexaose, lacto-N-neo-octaose, lacto-N-neo-  
decaose), characterized in that it also comprises  
a said enzyme chosen from  $\alpha$ -1,2-fucosyl-  
10 transferase and  $\alpha$ -1,3-fucosyl-transferase, and in  
that said cell also has a *WcaJ*<sup>-</sup> genotype and  
overexpresses the *RcsA* gene.
33. The method as claimed in claim 30, for the  
15 production of a sialyl and fucosyl derivative of  
lacto-N-neo-tetraose, lacto-N-neo-decaose,  
characterized in that it also comprises a said  
enzyme chosen from  $\alpha$ -2,3-sialyl-transferase and  $\alpha$ -  
2,6-sialyl-transferase, and also a said enzyme  
20 chosen from  $\alpha$ -1,2-fucosyl-transferase and  $\alpha$ -1,3-  
fucosyl-transferase, and in that said cell also  
has a *NanA*<sup>-</sup>, *NanT*<sup>+</sup>, *WcaJ*<sup>-</sup> genotype and  
overexpresses the *RcsA* gene and the gene for CMP-  
NeuAc-synthase.
- 25 34. The method as claimed in any one of claims 1 to  
28, for the production of 3'-sialyllactose ( $\alpha$ -  
NeuAc-[2 $\rightarrow$ 3]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-Glc) or 6'-  
sialyllactose ( $\alpha$ -NeuAc-[2 $\rightarrow$ 6]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]- $\beta$ -D-  
30 Glc), characterized in that:
- said cell is a bacterium of *LacZ*<sup>-</sup>, *LacY*<sup>+</sup>, *NanA*<sup>-</sup>  
or *NanT*<sup>+</sup> genotype;
  - said enzymes are CMP-NeuAc-synthase and  $\alpha$ -2,3-  
sialyl-transferase or  $\alpha$ -2,6-sialyl-transferase;
  - 35 • said substrate is glycerol;
  - said inducer is isopropyl- $\beta$ -D-thiogalactoside  
(IPTG);
  - said precursors are lactose and sialic acid.

35. The method as claimed in any one of claims 1 to 28, for the production of 3'-fucosyllactose ( $\beta$ -D-Gal-[1 $\rightarrow$ 4]-( $\alpha$ -L-Fuc-[1 $\rightarrow$ 3]-D-Glc) or 2'-fucosyllactose,  $\alpha$ -L-Fuc-[1 $\rightarrow$ 2]- $\beta$ -D-Gal-[1 $\rightarrow$ 4]-D-Glc characterized in that it comprises a said enzyme chosen from  $\alpha$ -1,3-fucosyl-transferase or  $\alpha$ -1,2-fucosyl-transferase, and in that the cell has a *wcaj<sup>-</sup> lacZ<sup>-</sup>* genotype and overexpresses the *rcsA* gene and in that said precursor is lactose.
36. The method as claimed in any one of claims 1 to 28, for the production of allyl 3-O-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl)- $\beta$ -D-galactopyranoside, ( $\beta$ -D-GlcNac-[1 $\rightarrow$ 3]- $\beta$ -D-Gal-1 $\rightarrow$ O-allyl), characterized in that:
- said cell is a bacterium of *LacZ<sup>-</sup>, LacY<sup>+</sup>* genotype;
  - said enzyme is  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase;
  - said substrate is glycerol;
  - said inducer is isopropyl  $\beta$ -D-thiogalactoside (IPTG);
  - said precursor is allyl- $\beta$ -D-galactopyranoside.
37. The method as claimed in any one of claims 1 to 28, for the production of analogs of lacto-N-neotetraose and of polylactosamines in which the glucose residue is replaced with an allyl group, characterized in that
- said cell is a bacterium of *LacZ<sup>-</sup>, LacY<sup>+</sup>* genotype;
  - said enzymes are  $\beta$ -1,3-N-acetyl-glucosaminyl-transferase and  $\beta$ -1,4-galactosyl-transferase;
  - said substrate is glucose;
  - said inducer is isopropyl  $\beta$ -D-thiogalactoside (IPTG);
  - said precursor is allyl- $\beta$ -D-galactopyranoside.

38. The method as claimed in one of claims 31 to 35,  
for the production of oligosaccharide analogs in  
which the glucose residue is replaced with an  
allyl group, characterized in that said precursor  
is allyl- $\beta$ -D-galactoside.
39. The method as claimed in claims 1 to 38, for  
producing an oligosaccharide labeled with at least  
one isotope, characterized in that said cell is  
cultured on said carbon-based substrate labeled  
with said isotope and/or in the presence of a said  
precursor labeled with said isotope.
40. An oligosaccharide which may be obtained by the  
method as claimed in any one of claims 1 to 39.
41. An oligosaccharide which may be obtained by the  
method as claimed in any one of claims 17, 19, 36  
and 37, characterized in that the double bond of  
the allyl group of said oligosaccharides is  
chemically modified by addition, oxidation or  
ozonolysis reactions to form activated  
oligosaccharides that may be used for the chemical  
synthesis of glycoconjugates or glycopolymers.
42. The oligosaccharide as claimed in claim 40 or 41,  
as a medicinal product.
43. The oligosaccharide as claimed in claim 42, as a  
medicinal product intended to selectively prevent  
the adhesion of biological molecules.
44. The oligosaccharide as claimed in claim 42, as a  
medicinal product intended for treating cancer,  
inflammation, heart diseases, diabetes, bacterial  
infections, viral infections and neurological  
diseases and grafts.



45. A pharmaceutical composition, characterized in  
that it comprises an oligosaccharide as claimed in  
any one of claims 42 to 44 and a pharmaceutically  
acceptable vehicle.

46. The agricultural or agronomic use of an  
oligosaccharide as claimed in claim 40 or 41,  
especially for the growth and defense of plants.

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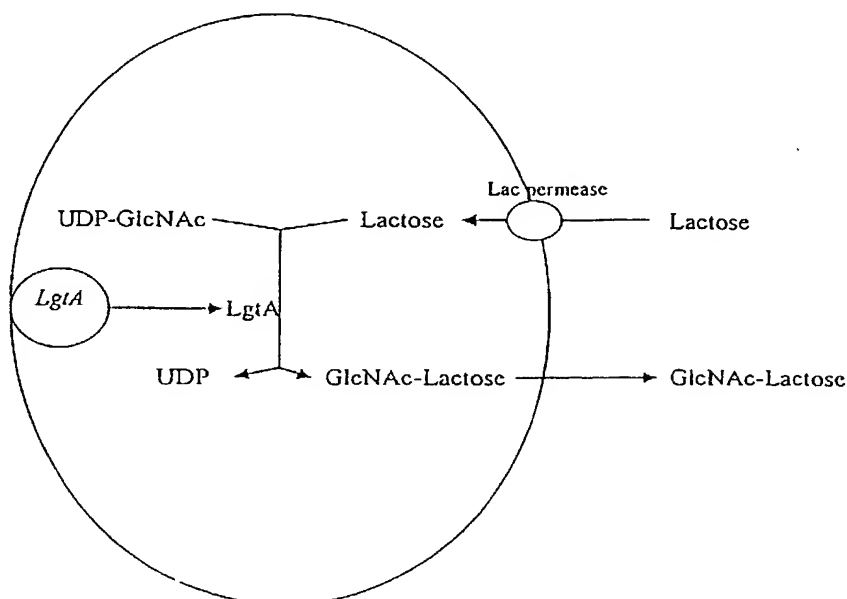
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[Suite sur la page suivante]

(54) Title: METHOD FOR PRODUCING OLIGOPOLYSACCHARIDES

(54) Titre: PROCEDE DE PRODUCTION D'OLIGOSACCHARIDES



(57) Abstract: The invention concerns the production by microbiological process of oligopolysaccharides of biological interest. More particularly, the invention concerns a method for synthesizing *in vivo* oligopolysaccharides by internalization of an exogenous precursor in growing bacterial cells expressing adequate modifying and glycosylating genes.

[Suite sur la page suivante]

WO 01/04341 A1

Title: METHOD FOR PRODUCING  
OLIGOPOLYSACCARIDES

Inventor(s): SAMAIN, Eric et al.

Appl. No.: 10/019,954

WO 01/04341

PCT/FR00/01972

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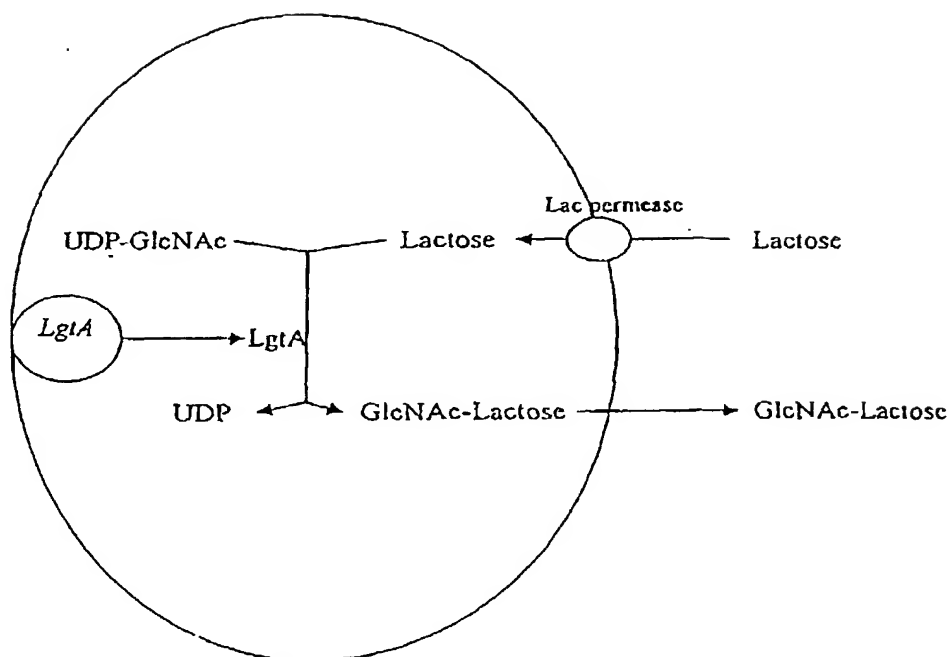


FIG-1

Title: METHOD FOR PRODUCING  
OLIGOPOLYSACCARIDES

Inventor(s): SAMAIN, Eric et al.

Appl. No.: 10/019,954

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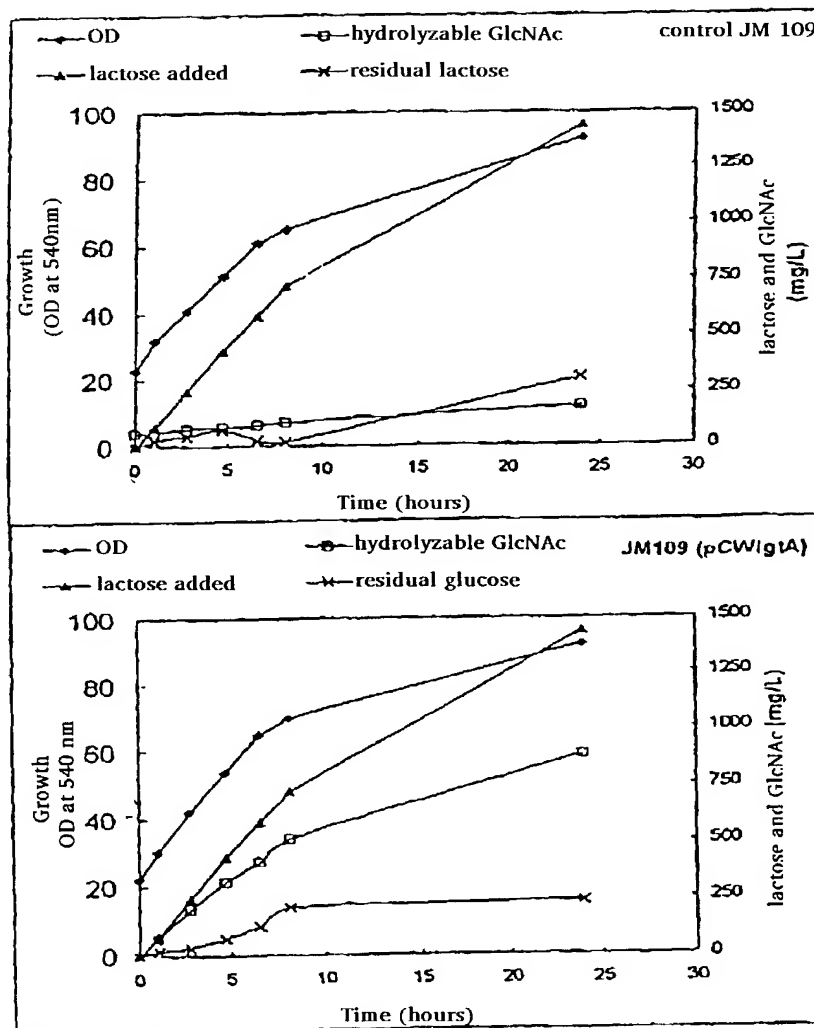


FIG-2

Title: METHOD FOR PRODUCING  
OLIGOPOLYSACCARIDES

Inventor(s): SAMAIN, Eric et al.

Appl. No.: 10/019,954

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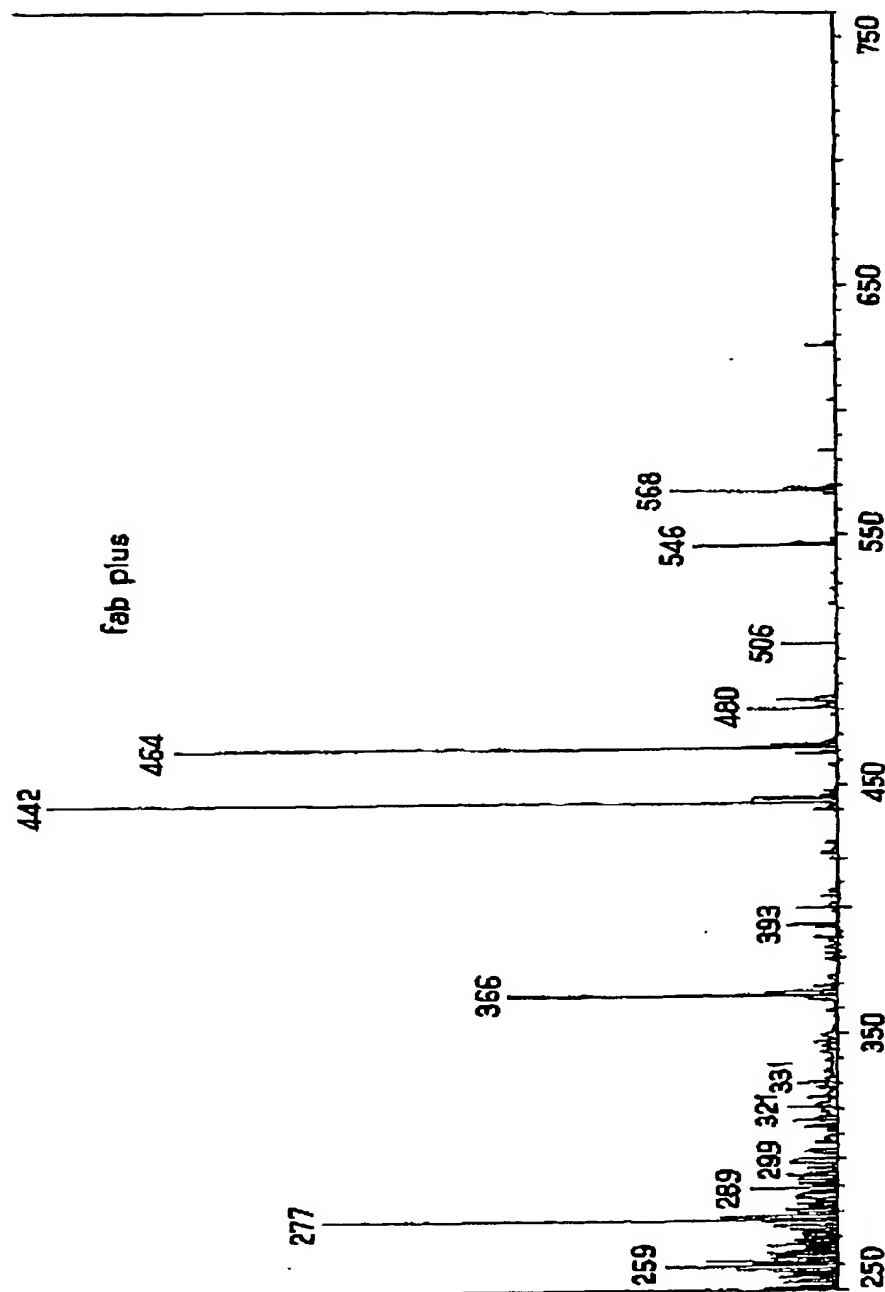


FIG. 3

Title: METHOD FOR PRODUCING  
OLIGOPOLYSACCARIDES

WO 00/04341

Inventor(s): SAMAIN, Eric et al.  
Appl. No.: 10/019,954

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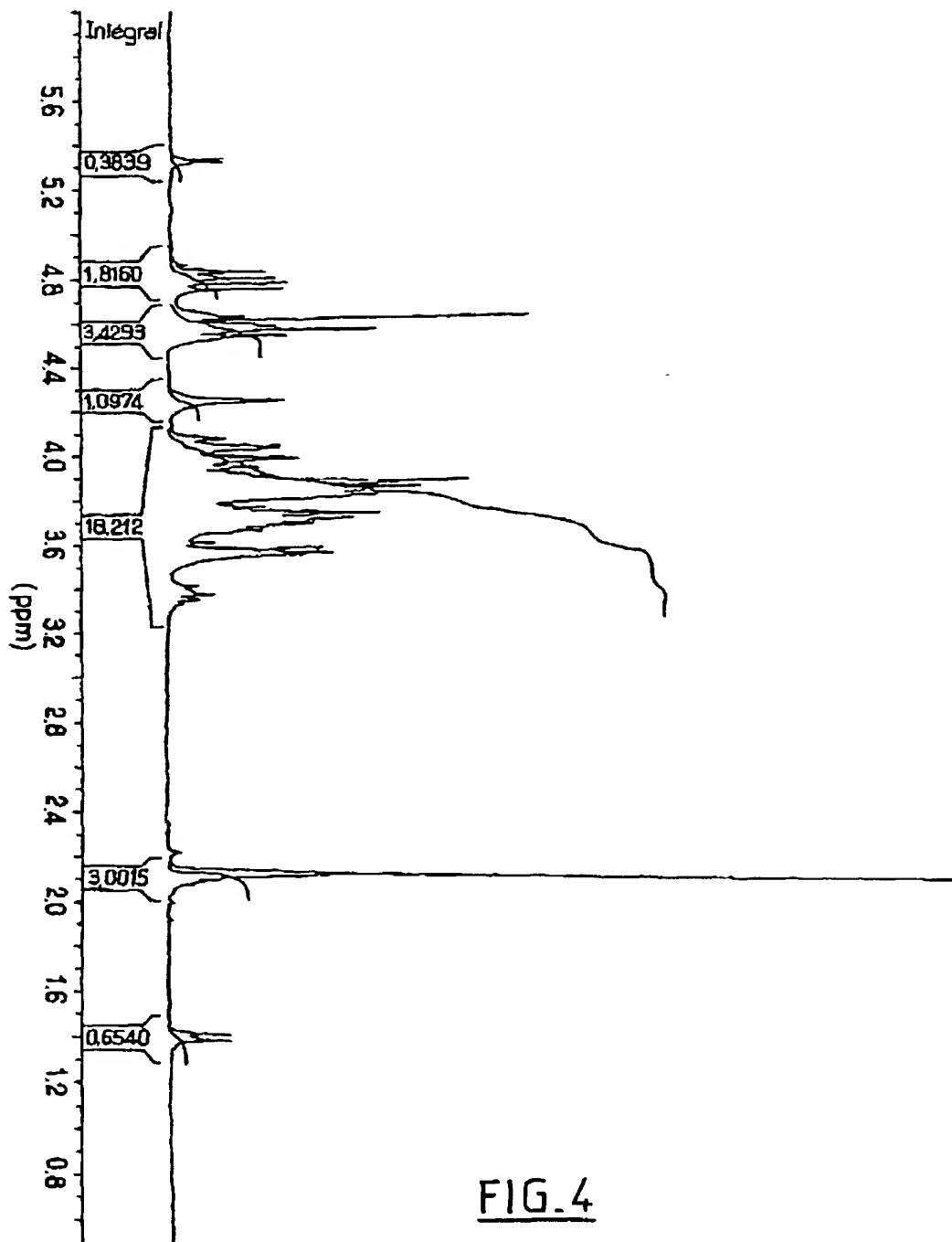


FIG.4

Title: METHOD FOR PRODUCING  
OLIGOPOLYSACCARIDES

Inventor(s): SAMAIN, Eric et al.

Appl. No.: 10/019,954

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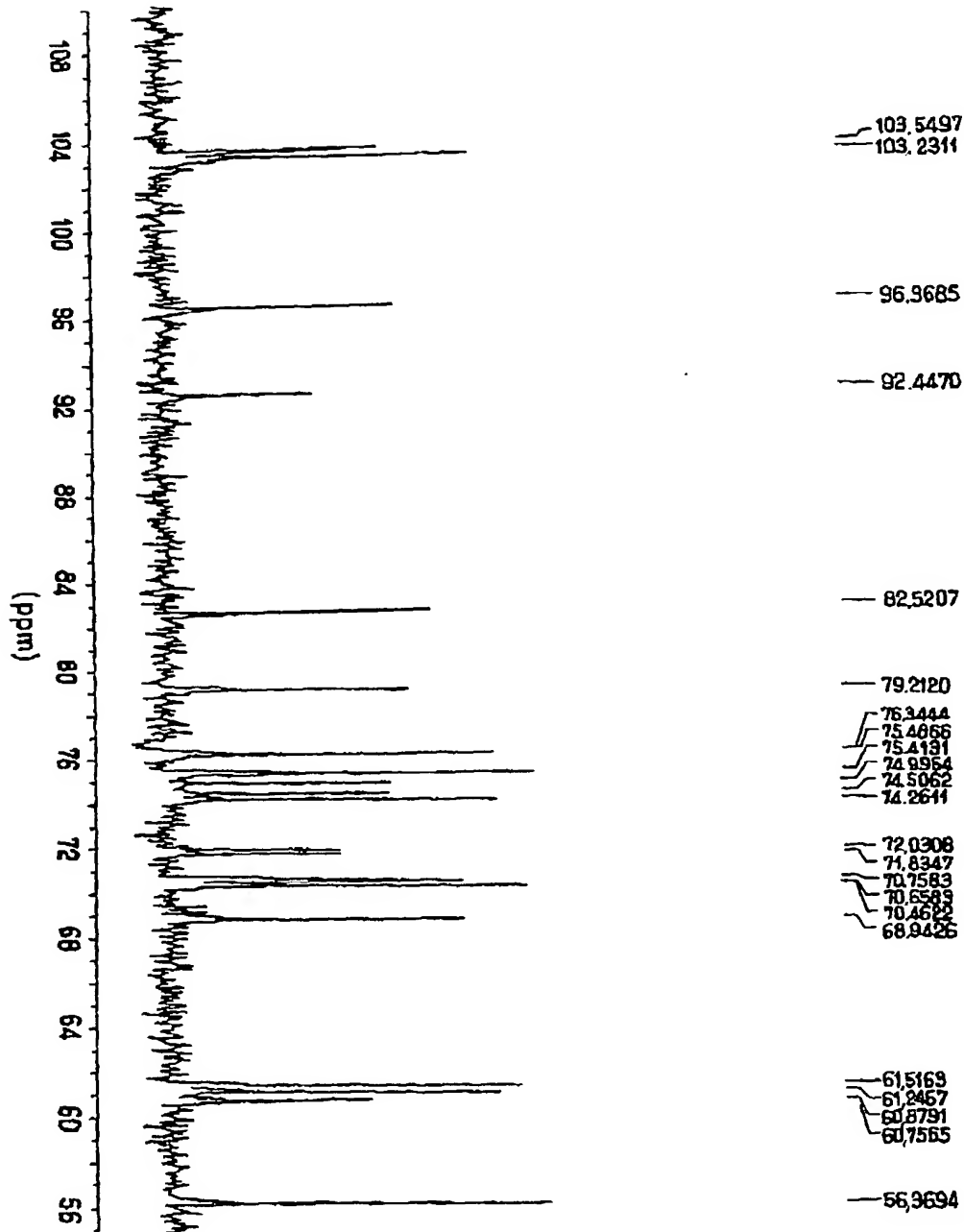


FIG. 5

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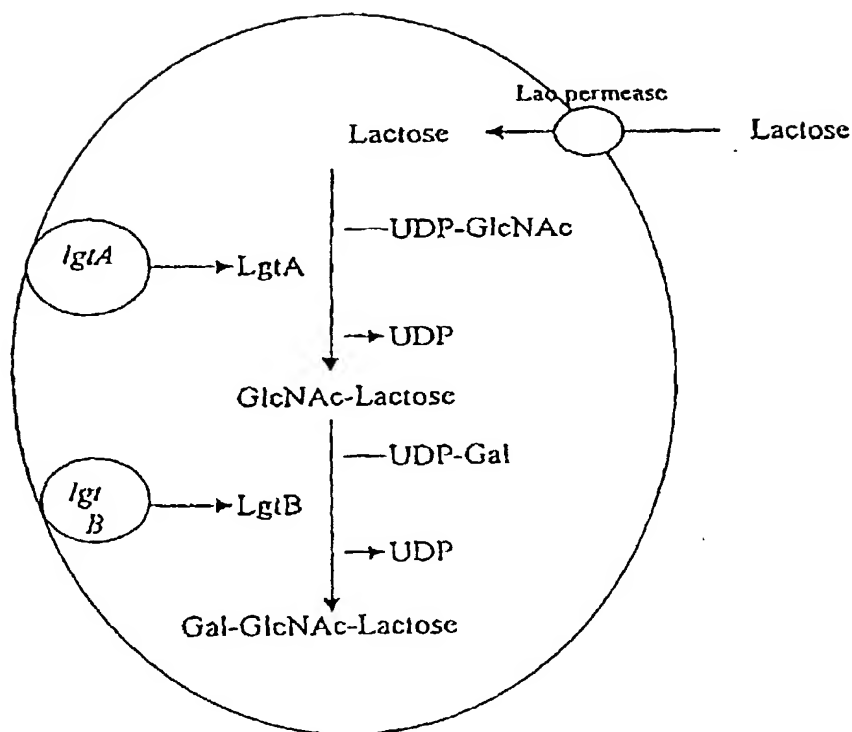


FIG-6



Title: METHOD FOR PRODUCING  
OLIGOPOLYSACCHARIDES

Inventor(s): SAMAIN, Eric et al.

Appl. No.: 10/019,954

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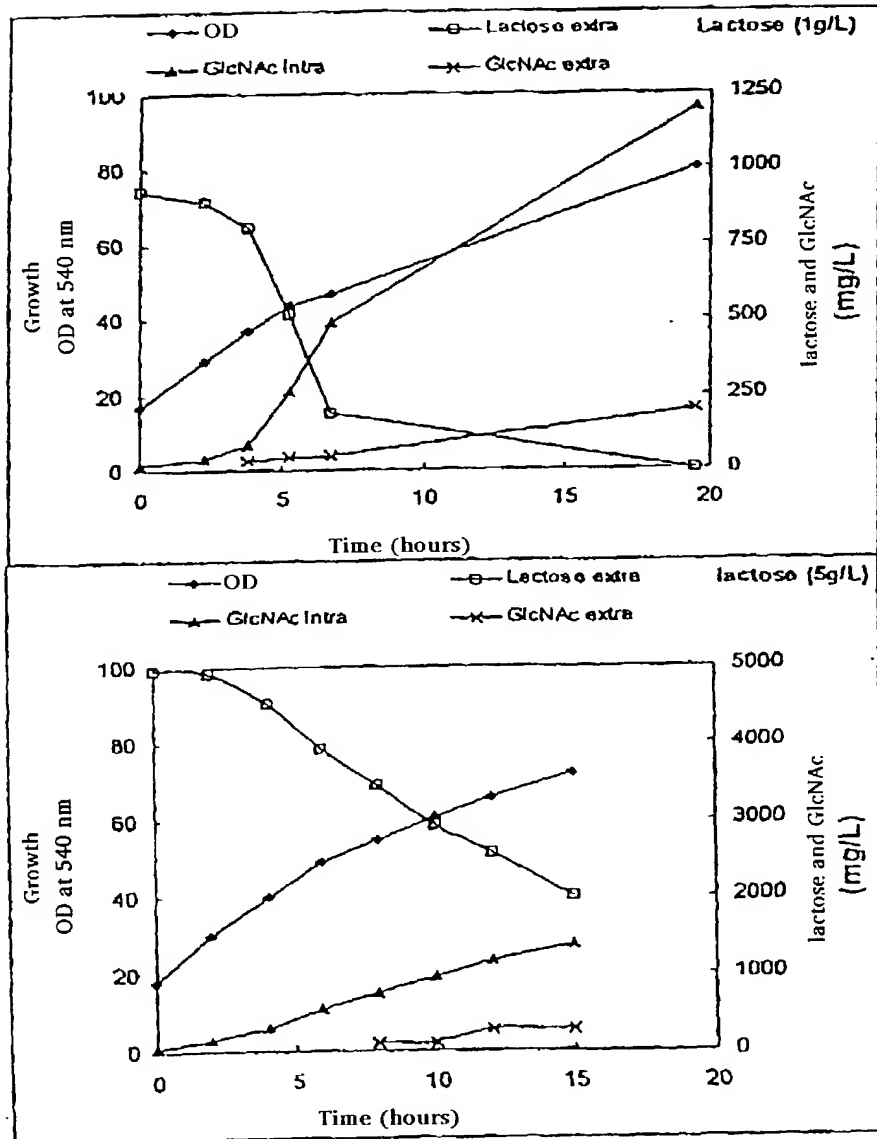


FIG-7

Title: METHOD FOR PRODUCING  
OLIGOPOLYSACCHARIDES

Inventor(s): SAMAIN, Eric et al.

Appl. No.: 10/019,954

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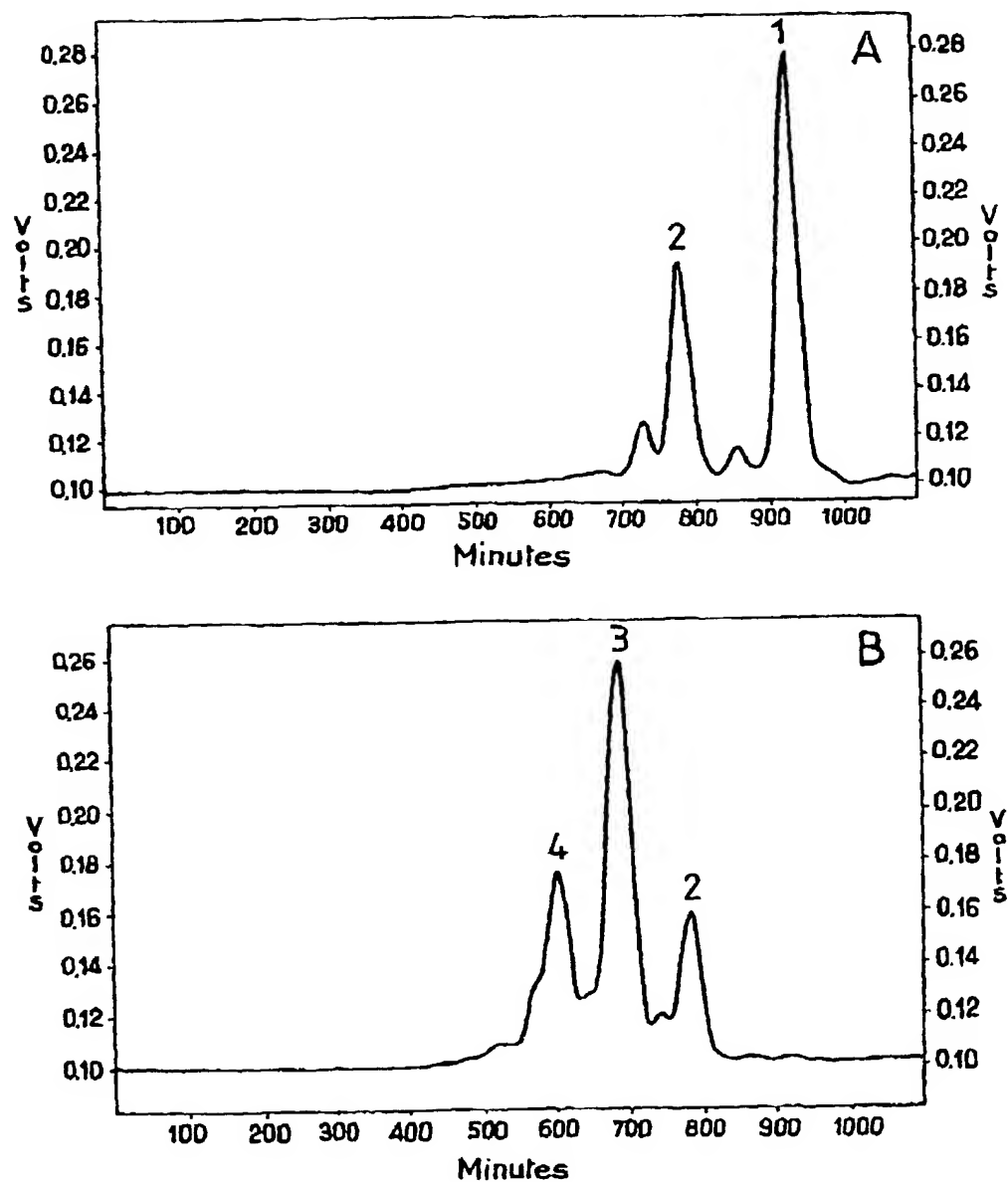


FIG. 8

Title: METHOD FOR PRODUCING  
OLIGOPOLYSACCARIDES

Inventor(s): SAMAIN, Eric et al.

Appl. No.: 10/019,954

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PCT/FR00/01972

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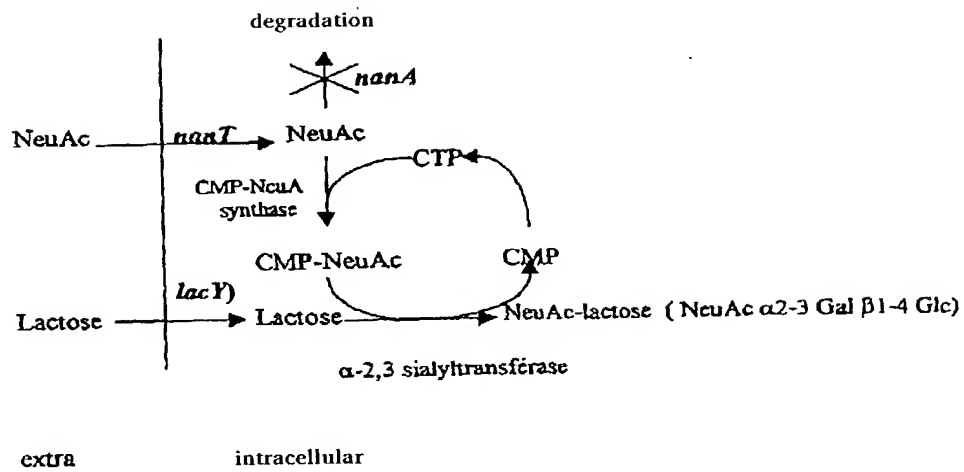


FIG-9

**DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I HEREBY DECLARE:

THAT my residence, post office address, and citizenship are as stated below next to my name;

THAT I believe I am the original, first, and sole inventor (if only one inventor is named below) or an original, first, and joint inventor (if plural inventors are named below or in an attached Declaration) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**METHOD FOR PRODUCING OLIGOPOLYSACCARIDES**

**(Attorney Docket No. 065691-0267)**

the specification of which (check one)

       is attached hereto.

  X   was filed on July 7, 2000 as United States Application Number or PCT International Application Number PCT/FR00/01972 and was amended on        (if applicable).

THAT I do not know and do not believe that the same invention was ever known or used by others in the United States of America, or was patented or described in any printed publication in any country, before I (we) invented it;

THAT I do not know and do not believe that the same invention was patented or described in any printed publication in any country, or in public use or on sale in the United States of America, for more than one year prior to the filing date of this United States application;

THAT I do not know and do not believe that the same invention was first patented or made the subject of an inventor's certificate that issued in any country foreign to the United States of America before the filing date of this United States application if the foreign application was filed by me (us), or by my (our) legal representatives or assigns, more than twelve months (six months for design patents) prior to the filing date of this United States application;

THAT I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment specifically referred to above;

THAT I believe that the above-identified specification contains a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention, and sets forth the best mode contemplated by me of carrying out the invention; and

THAT I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I HEREBY CLAIM foreign priority benefits under Title 35, United States Code § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number	Country	Foreign Filing Date	Priority Claimed?	Certified Copy Attached?
99 08 772	France	07/07/1999	YES	

I HEREBY CLAIM the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

U.S. Provisional Application Number	Filing Date

I HEREBY CLAIM the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Application Number	Parent Filing Date	Parent Patent Number

I HEREBY APPOINT the registered attorneys and agents at Customer Number 22428



to have full power to prosecute this application and any continuations, divisions, reissues, and reexaminations thereof, to receive the patent, and to transact all business in the United States Patent and Trademark Office connected therewith.

I request that all correspondence be directed to:

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22428

PATENT TRADEMARK OFFICE

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I UNDERSTAND AND AGREE THAT the foregoing attorneys and agents appointed by me to prosecute this application do not personally represent me or my legal interests, but instead represent the interests of the legal owner(s) of the invention described in this application.

I FURTHER DECLARE THAT all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1-0  
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